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(54) Title: HEPATITIS B VIRUS MUTATIONS (57) Abstract <p>The present invention relates to hepatitis B virus (HBV) disease and in particular provides polynucleotide sequences which are characteristic of certain disease states. Said HBV polynucleotides are for use in evaluation of a HBV disease state which comprises at least two of the following: (i) a mutation in the Enhancer I region; (ii) a mutation in the Negative Regulatory Element region; (iii) a mutation in the Enhancer II/Core Upstream Regulatory Sequence/Basal Core Promoter region; and (iv) a mutation which leads to an X peptide amino acid change to provide a cysteine or methionine residue; the mutation being a variation from the normal nucleotide at that position in a respective one of HBV genotypes A to F. The invention also provides tests for detecting the polynucleotides. A test involving protein binding interactions with the polynucleotides is also provided.</p>		

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HEPATITIS B VIRUS MUTATIONS

The present invention relates to hepatitis B virus (HBV) disease, and in-particular provides polynucleotide sequences which are characteristic of certain disease states. The invention also provides tests for detecting the polynucleotides. A test involving protein binding interactions with the polynucleotides is also provided.

Fulminant hepatitis B [FHBV] is a rare but serious complication of acute infection, associated with massive hepatocellular necrosis and mortality rates exceeding 50%, depending on coma grade ^{1,2,3}. FHBV pathogenesis has been associated with elevated rates of viral replication ^{4,5} and/or abnormally rapid clearance.

There has been considerable debate as to the relative role of host versus viral factors in the aetiology of FHBV. Outbreaks have been traced to common sources ^{5,6,7,8}, suggesting the involvement of specific viral strains. Furthermore, particular viral variants have been implicated, such as the precore stop variant A₁₈₉₆ ^{5,6,9,10}, which inhibits production of HBeAg, a protein believed to have immunomodulatory effects ^{11,12,13} and which may exert a dominant negative effect on HBV replication ¹⁴. An association has also been found between FHBV and variants in the basal core promoter (BCP)¹⁵ (Figure 1), which regulates the production of precore and pregenomic RNAs; in particular, T₁₇₆₂ and A₁₇₆₄ ^{16,17} have been implicated. However, as with A₁₈₉₆, these variants have also been found in non-FHBV sequences and do not occur in all cases of FHBV ^{18,19,20}. Complicating the issue, index case and contact pairs have been identified where the complete viral genomes are identical, or nearly so ^{21,22}. One study showed division of patients with FHB into separate classes - rapid (<1-2 weeks) and slow (> 3weeks) - according to rates of clearance of HBsAg and HBV DNA and intervals between onset of clinical illness and hepatic encephalopathy ²³.

We have tested the hypothesis that there are common patterns of genetic variation or 'motifs' among FHBV strains. Using statistical and phylogenetic analyses of the X and C genes from unrelated FHBV cases and contacts, of which 19 are previously unreported sequences, and sequence information from enhancer I region, we show that there are strains of HBV associated with this disease, each with a distinct variant pattern, effect on mortality and speed of progression to FHBV symptoms.

Thus, we have reviewed HBV sequences associated with fulminant hepatitis disease in a number of patients. We have been unable to associate any single mutation with the fulminant form of the disease. According to the present invention, by phylogenetic analysis we have surprisingly found that certain combinations of unusual nucleotide substitutions (not only relative to the known

5 respective genotype but which are sometimes also unusual in all genotypes) in particular positions in the sequence correlate strongly with FHBV disease. The mutations may not in themselves be novel, but in combination in the respective genotype background they are important. It also appears that a variety of mutation combinations can lead to a common phenotype. The combinations of particular nucleotides in certain positions are referred to as "motifs" herein.

10 The present invention in one aspect provides hepatitis B virus polynucleotide for use in evaluation of a hepatitis B disease state which comprises at least two of the following:

- (i) a mutation in the Enhancer I region;
- (ii) a mutation in the Negative Regulatory Element region;
- (iii) a mutation in the Enhancer II/Core Upstream Regulatory Sequence/Basal Core Promoter
15 region; and
- (iv) a mutation which leads to an X peptide amino acid change to provide a cysteine or methionine residue;

the mutation being a variation from the normal nucleotide at that position in a respective one of HBV genotypes A to F. There may be one or more mutations in any of (i) to (iv).

20 The hepatitis disease state which may be evaluated according to the present invention include fulminant hepatitis, severe chronic hepatitis, and serologically unusual forms of hepatitis (including HBsAg negative infection, anti-HBcore negative infection and serologically negative hepatitis - all of which have been linked to variation in the X peptide or the other regions involved in transcriptional control as set out above).

25 Another aspect of the invention provides hepatitis B virus polynucleotide for use in detection of fulminant hepatitis B viral infection which comprises at least two of the following:

- (i) a mutation at one or more of the following positions 1050, 1249 and 1250;
- (ii) a mutation at one or more of the following positions 1633 and 1634;
- (iii) a mutation at one or more of the following positions 1653, 1754, 1762, 1764, 1766, 1768,
30 1809, 1821, 1826 and 1838/9 insertion;
- (iv) a mutation which leads to an X-peptide change to provide cysteine or methionine at one or more of amino acid positions 26, 72, 88, 127 and 130;

the mutations being variations from the normal nucleotide at that position in a respective one of HBV genotypes A to F. On transmission to a contact these sequences may result in fulminant hepatitis B.

35 The polynucleotide sequences may be DNA or RNA sequences. Sequences are given in the 5' to 3' direction. Sequence numbering is according to the convention established by Galibert, and

5 numbers from the unique ECoR1 restriction site of HBV. The polynucleotide sequence need not be continuous and may be broken up into contiguous or non-contiguous segments. However, the segments will, of course, have a common origin.

The polynucleotides and peptides useful for the present invention are generally in isolated form, isolated from materials from the host patient, and usually isolated from other material of HBV
10 origin.

Generally, the polynucleotides are fragments of the complete genome e.g. 20-1000 nucleotides in length, particularly 50-500, especially 100-200. The term 'fragment' means a polynucleotide of length less than the corresponding HBV genotype genome. X peptide fragments are usually less than the length of the naturally occurring gene expression product.

15 Certain polynucleotides and peptides are novel and these form a further aspect of the invention.

The hepatitis disease state may be determined directly by sequencing an HBV sample from the patient. More conveniently, the polynucleotide motifs may be detected using hybridisation probes of complementary sequence which are capable of binding thereto under appropriate stringency
20 conditions (as may be determined readily by experimentation). Generally each probe will be directed to a portion of the sequence containing a single mutation. However, probes directed against multiple mutations may also be used. The probes may be labelled with conventional labels, such as radiolabels, fluorescence labels etc. A particular test format comprises a solid substrate having thereon a series of bands of characteristic hybridisation probes. The pattern of binding of an HBV sample from a
25 patient to the bands enables a determination of the presence or absence of motifs characteristic of a particular disease state to be made.

Another test format for the detection of polynucleotide motifs characteristic of FHBV involves conducting polymerase chain reaction (PCR) using primers having a sequence complementary to the FHBV characteristic sequences given above.

30 We have confirmed that certain nucleotide motifs in the NRE/BCP/Enhancer II region (a region having gene expression control properties) lead to enhanced gene expression in an experimental plasmid (containing a luciferase gene under control of said motif-containing HBV enhancer region). Enhanced expression and replication of HBV virus in vivo is postulated to lead to the acute fulminant HBV disease state.

35 Without wishing to be bound by theory, it is hypothesised that enhanced gene expression resulting from the motif-containing sequences is due to a reduction of the ability of the motif-

5 containing control sequences to bind inhibitory proteins present in host cells (i.e. the patient). Normal inhibition of HBV gene expression is thus reduced. Experimental evidence of altered nucleotide-host protein binding is given herein.

Thus, the invention also provides a test method for determining binding interaction between host or viral proteins and HBV regulatory polynucleotides.

10 The invention also provides certain antigenic X-peptides, antibodies (e.g. monoclonal) thereto, and immunoassays.

Embodiments of the invention will now be described by way of example only. Sections I and II are devoted to phylogenetic analysis and increased transcription respectively.

SECTION I

15 Statistical and phylogenetic analysis was carried out as follows, to show that certain mutational motifs were related to FHBV.

METHODS

Patients and Sequence Data

Because previous studies have shown that sequences from index and chronic contacts of
20 FHBV patients are often genetically identical ^{21,22}, all chronic [CHBV] and acute [AHBV] contact cases were treated as FHBV. For the C gene (nucleotide positions 1814-2458) viral sequences from 30 patients linked to FHBV, representing 27 unlinked FHBV episodes, were analysed. Of these 30, 19 (16 FHBV, 2 CHBV, 1 AHBV) are newly reported here and 11 (6 FHBV, 5 CHBV) were obtained from the GenBank/EMBL/DDBJ sequence databases. For the X gene (nucleotide positions
25 1374-1838) 26 FHBV sequences representing 25 unlinked FHBV episodes were analysed. Of these 26, 17 (14 FHBV, 2 CHBV, 1 AHBV) are newly reported here and 9 (6 FHBV, 3 CHBV) were obtained from the sequence databases. With respect to the non-FHBV sequences, 2 X and C gene sequences are newly reported here while 159 complete C gene sequences and 86 complete X gene sequences were obtained from the sequence databases or from a recent study ¹⁹ (sequence list
30 available from authors). This produced total datasets of 191 C gene and 114 X gene sequences. All unpublished sequences have been submitted to GenBank and have been assigned accession numbers xxxx - yyyy.

Fulminant patient information is detailed in Table 1. Patients were classified as either 'rapid' or 'slow' progressors to FHBV symptoms (hepatic encephalopathy). Rapid progressors were those
35 who progressed from stage 0 to stage 4 coma within a week. Sequences from contact cases first

5 reported here include two symptomless anti-HBe positive carriers (sequences 10 and 21) who fatally infected two sexual partners each ⁶, and one of a pair of sisters, infected simultaneously, one of whom developed mild acute infection (sequence 7) while the other acquired severe FHBV (sequence 6). 2 patients were serum anti-HDV IgM positive and thus co-infected with hepatitis D virus. None of our patients were positive for anti-HCV antibodies. In order to assess the incidence of variation in
10 enhancer I a GenBank/ncbi/BLAST search identified all published sequences in the region (74 sequences) (list available from authors).

DNA extraction and PCR amplification

DNA extraction, PCR amplification and sequencing were performed as previously described
27 (list of primers available from authors).

15 Phylogenetic Analysis

C and X gene sequences were aligned with the ClustalW program ²⁸ and phylogenetic trees constructed using a maximum likelihood method (program fastDNAm1 ²⁹). The maximum likelihood transition:transversion ratio (Ts/Tv) and relative rates of evolution for the three codon positions were estimated using a likelihood program (SPOT) ³⁰. The parameters so determined are listed in the figure
20 legends for Figures 2 and 3. In all cases unrooted phylogenetic trees were constructed and then midpoint rooted for clarity. For technical reasons, there was insufficient FHBV sequence information available for an analysis of FHBV sequence clustering in the enhancer I region.

Relative Rates of Evolution

In order to determine whether there was any difference in the rate of evolution between
25 FHBV and non-FHBV sequences, a relative rate test was undertaken (Figure 4). Distances between FHBV sequences (*a*) and their nearest non-FHBV neighbor (*b*) were compared to their nearest non-FHBV outgroup sequence (*c*) using the DNADIST (nucleotide) and PROTDIST (amino acid) programs within the PHYLIP package ³¹. 13 and 14 comparisons were made for the X and C genes, respectively. Tests were also performed for the antigenic regions in the C gene as defined in Carman
30 et al. 1995 ³² and according to the functional subdivisions in X identified in Yuh et al. 1992 ³³. Analysis at the protein level was limited statistically to the complete X and C genes and the combined antigenic and combined non-antigenic regions in C. Relative rate tests were also performed on FHBV sequences with and without the A₁₈₉₆ pre-core stop codon variant (9 and 7 comparisons, respectively) and on a set of non-FHBV A₁₈₉₆ cases (9 comparisons). In the tests involving A₁₈₉₆, sequences from
35 G₁₈₉₆ (HBeAg producing) CHBV cases acted as sequences *b* and *c*. In all cases the Wilcoxon Signed Rank Test was used to test for the significance of any differences in evolutionary rate.

5 Finally, variants were divided into those that altered the encoded amino acid (nonsynonymous changes) and those that did not (synonymous changes) and a comparison made between FHBV and non-FHBV cases using the program INA³⁴. The relative rate of synonymous and nonsynonymous change is a useful indicator of the strength of natural selection.

Analysis of Clustering

10 A cluster was defined here as an uninterrupted phylogenetic lineage of epidemiologically unlinked FHBV sequences. We undertook an analysis to determine whether FHBV sequences form distinct clusters on the tree more than expected by chance. This was done by treating FHBV status as an additional character state and calculating the expected number of evolutionary steps given the number of FHBV cases under a parsimony based model of evolutionary change³⁵, as implemented
15 in the program FMAB (Bollyky et al., submitted for publication).

RESULTS

Is there genetic similarity between epidemiologically unlinked FHBV cases? While visual inspection of the X gene (Figure 2) and C gene (Figure 3) phylogenetic trees suggests that FHBV sequences are clustered, it was necessary to prove this statistically. For the X gene, the distribution of the 25 independent FHBV sequences on the phylogenetic tree could be accounted for most parsimoniously by 14 unambiguous evolutionary steps, fewer than the minimum of 21 expected under a random model of sequence evolution ($P = 0.1 \times 10^{-5}$). In the C gene, the 17 observed unambiguous evolutionary steps for the 27 independent FHBV sequences was again less than the 25 expected if these sequences were not clustered ($P = 0.4 \times 10^{-7}$). This clustering reflects a high degree of genetic relatedness amongst FHBV sequences. Generally the same clusters were found in both genes, although sequences 15, 19, 21, 23 occupied different positions on the X and C gene trees. Whether these discrepancies reflect recombination events³⁶, as was clear in the case of non-clustered FHBV sequences 25 and 26, or the effect of rapid evolution associated here with FHBV sequences (see below), was unclear.

Do the clusters segregate according to sex, age, HBeAg status, viral genotype, or clinical outcome of their hosts? Table 1 details these associations. Clustering was most strongly linked to mortality, every cluster of sequences being uniform with respect to outcome (i.e. survival or death) with 2 exceptions, one was a liver transplant recipient and thus survived while the other was pregnant and died. All FHBV cases in individuals who were pregnant, hepatitis D virus co-infected, or over 30 years of age were fatal, with the exception of those patients who received liver transplants. Clustering was also linked to the speed of onset of hepatic encephalopathy and clearance of HBsAg and HBV DNA, with every cluster of sequences, with 2 exceptions, being uniform in this regard. The two patients who were co-infected with hepatitis D virus were found in the same cluster.

Are FHBV sequences characterised by different rates of nucleotide evolution and by changes in specific regions? Relative to their nearest non-FHBV phylogenetic neighbor, the FHBV sequences had a significantly higher rate of nucleotide evolution in both the X and C genes (Tables 2 and 3 respectively). In the X gene, this increase was also significant at the amino acid level, and localised to the BCP at both the nucleotide and amino acid levels. FHBV sequences also had a significantly higher number of synonymous changes in the X gene, indicating an elevated rate of background mutation. This relationship is also reflected in the codon position specific weighting ratios determined for the X gene phylogenetic tree, where first and second codon position changes occur at an elevated rate relative to third position changes. In the C gene, the elevated rate of evolution in FHBV

sequences was significant at the nucleotide level for the whole gene and for non-antigenic regions.

Are particular variants associated with FHBV sequences and clusters of sequences? No single unique variant linked all individual FHBV sequences or groups of sequences; this has been reported previously^{15, 20, 24}. However, particular variants characterised FHBV sequences and clusters of sequences (detailed in Table 4). On the nucleotide level, these included variants in enhancer I (enhancer I is known to stimulate transcriptional function of both the X gene promoter and the BCP³⁷), variants in the negative regulatory element (NRE)³⁸, and the BCP region of enhancer II. On the amino acid level, these included uncharacteristic patterns of otherwise highly conserved cysteine residues³⁹, which would be expected to play a central role in determining the tertiary structure of protein products of X, and aberrant methionine residues, which might be expected to alter levels of the three protein products thought to be coded by the X gene⁴⁰ and thus have effects on transcriptional transactivation of HBV.

Many of these nucleotide and protein variants are genotype specific, altering nucleotides otherwise typical for particular genotypes; these relationships are detailed in Table 5. Among variants which have been previously suggested to play a role in FHBV pathogenesis, T₁₆₇₆ and A₁₇₅₇,¹⁵ were found to be characteristic of genotype D; 30 and 21 of the 35 genotype D sequences in our dataset possessed these variants, respectively, and so they were not associated with FHBV.

Were there particular 'motifs', or combinations of variants, which were unique to FHBV? The FHBV sequences possess particular motifs, or combinations of variants which distinguish them from non-FHBV sequences. The combination of either aberrant methionine residues, aberrant cysteine residues, or one of three identified nucleotide variants in enhancer I with any of a group of notable variants in the NRE, the BCP, or the A₁₈₉₆ pre-core stop variant was nearly exclusive to FHBV sequences (Table 6). T₁₇₆₂ and A₁₇₆₄^{16, 17} characterised some clusters of FHBV sequences but were not by themselves disproportionately represented in FHBV cases. However, where both these two variants are present with either T₁₇₆₆ or A₁₇₆₈, 3 of 4 examples were FHBV. With a single exception, whenever the A₁₈₉₆ variant was found with the aberrant cysteine and methionine residues or enhancer I variants described here it was in a sequence associated with FHBV.

What effect does the A₁₈₉₆ precore stop variant have on the rate and pattern of evolution in FHBV sequences? In G₁₈₉₆ FHBV sequences and in the complete FHBV dataset (A₁₈₉₆ plus G₁₈₉₆ FHBV sequences), changes in the complete C gene and in non-antigenic regions accumulated faster than in non-FHBV sequences. In contrast, A₁₈₉₆ FHBV sequences had significantly higher rates of nucleotide evolution than in G₁₈₉₆ non-FHBV sequences in the complete C gene and in both non-

antigenic and antigenic regions, particularly within the anti-HBc / e2 and anti-HBc / e3 epitopes, as well as significantly higher rates of amino acid evolution in all areas tested. These results for A₁₈₉₆ FHBV also differed from the CHBV A₁₈₉₆ controls, which did not accumulate changes at a significantly increased rate in non-antigenic regions and differed in the specific antigenic regions affected (Table 3).

TABLE LEGENDS.

TABLE 1: Clinical data and clustering of FHBV cases and contacts.

A cluster was defined as an uninterrupted phylogenetic lineage of epidemiologically unlinked FHBV sequences. Occasionally there were minor discrepancies in the clusters defined in the X and C genes due to lack of phylogenetic resolution. In these cases, such as the movement of sequence 19 between clusters 1 and 6, weight was given to the positioning seen within the X gene, as this is the gene where the majority of the variants associated with FHBV are described. Those sequences which do not cluster in the X gene but do in the C gene, either because those sequences were unavailable for the X gene or because of the factors such as recombination or the rapid evolution associated here with FHBV, are marked with a *. Sequences 25 and 26 are epidemiologically linked and therefore are not numbered as a cluster. Individual sequences are those which do not cluster with other fulminant sequences in either gene.

C,X= Core and X genes. M=Male,F=Female.FHBV= Fulminant hepatitis B virus infection; AHBV= Acute hepatitis B virus infection; CHBV = Chronic hepatitis B virus infection.[§] Reported here. **For chronic contacts, the outcome and course listed are those of the fulminant index cases associated with that contact case.@ Patient was pregnant.

* Patient received liver transplant. β Patient was hepatitis D virus co-infected. § These sequences show evidence of recombination, grouping with D genotype sequences in the C gene and with A genotype sequences in the X gene.

TABLE 2: Relative rate tests for the X gene comparing rates of evolution between FHBV and non-FHBV sequences.

All results are expressed as *P*= the probability that FHBV sequences are evolving faster than non-FHBV sequences. Significant results are in bold type.* Nucleotides are numbered from the unique

EcoR1 site.* Amino acids are numbered from the start of the X gene. CURS= Core Upstream Regulatory Sequence, BCP= Basal Core Promoter. Relative rate test results for synonymous changes were significantly different only for the number of synonymous changes in the X gene between FHBV and non-FHBV sequences ($P=0.014$).

TABLE 3:

Relative rates tests for the C gene comparing rates of evolution in the complete FHBV dataset, A₁₈₉₆ FHBV variants, G₁₈₉₆ FHBV variants, and non-FHBV A₁₈₉₆ variants, all against G₁₈₉₆ non-FHBV sequences.

All results are expressed as P = the probability that FHBV sequences are evolving faster than non-FHBV sequences. Significant results are in bold type. * Nucleotides are numbered from the unique EcoR1 site. Relative rate tests for synonymous and nonsynonymous changes were not significantly different between FHBV and non-FHBV sequences in any areas tested.

TABLE 4: Variants suggested to play a role in FHBV and their distribution in the dataset.

BCP= Basal Core Promoter, NRE= Negative Regulatory Element, Pre-C = Precore region; A₁₈₉₆= premature stop codon that inhibits HbeAg production.

* There were a number of variants in the BCP/enhancer II complex whose incidence was unique to a single sequence or pair of sequences. These were: A₁₇₇₉(CHBV1), A₁₇₉₀(CHBV1), A₁₇₉₄(CHBV1), T₁₈₁₀(HPBC5HK02, HPBC4HST2), T₁₈₁₁(HPBC5HK02,HPBC4HST2), C₁₈₁₉(FHBV13), C₁₈₂₁(CHBV1), and A₁₈₂₆(FHBV1).

± Variation related to FHBV at these sites involves deviations from otherwise highly conserved genotype nucleotide identity. More than one nucleotide variant therefore describes the relevant variation at this position (Table 5). § Where applicable. += That variant seen, -= Other variant seen at that position, NA= Not available.** Sequences FHBV15 and CHBV1 have insertions at position 1838 which cause a frame shift.

TABLE 5: Combinations of variants in motifs that may play role in FHBV cases.

Variants or combinations of variants playing role in FHBV. += variants observed. -= variants not observed. n/a = not available. ?= not known. Underlined variants are unique variants observed in that particular case or only few cases. Bold variants occurred outside the genotype context. C₁₇₄₀ is not a unique variant but significant in combination with another unusual variant (c₁₇₇₃). * = For case

- 5 FHBV-10, no variants were identified and for case FHBV-11, sequence data was not available in the regions considered except pre-core. Enh=enhancer. BCP= basal core promoter. NRE=negative regulatory element. CURS= core upstream regulatory element.

TABLE 6: Deviations from typical genotype associations at a number of particular nucleotide and amino acid positions in the X gene.

- 10 * All three occurrences of C₁₇₇₃ in genotype A are in FHBV sequences. Variation in the other genotype at this location seems to be less restrictive.** Sequences either have a cysteine a position 6 or position 78, depending on genotype. The cysteine at position 78 has been shown to be disulphide bonded³⁰, suggesting this difference is of some importance in determining protein structure.

TABLE 7: Motifs, or combinations of variants, suggested to play a role in FHBV and their distribution in the dataset.

- 15 MOTIF 1= BCP nucleotide variant+ aberrant cysteine residue in X protein.
 MOTIF2 = BCP nucleotide variant + aberrant methionine residue in X protein.
 MOTIF 3 = NRE nucleotide variant + aberrant cysteine residue in X protein.
 MOTIF 4 = NRE nucleotide variant + aberrant methionine residue in X protein.
 20 MOTIF 5 = NRE nucleotide variant + enhancer I nucleotide variant.
 MOTIF 6 = A₁₈₉₆ precore stop variant + aberrant cysteine residue in X protein.
 MOTIF 7 = A₁₈₉₆ precore stop variant + aberrant methionine residue in X protein.
 MOTIF 8 = A₁₈₉₆ precore stop variant + enhancer I nucleotide variant.
 BCP = Basal Core Promoter, NRE= Negative Regulatory Element, Pre-C= Precore region; A₁₈₉₆=
 25 premature stop codon that inhibits HbeAg production. Only sequences for which complete information was available for the areas in question are listed. The A₁₈₉₆ total is out of 43 X gene sequences with the A₁₈₉₆ precore stop variant.

TABLE 8: FHBV Variant Motifs

- * See section on genotype specific FHBV variants.

- 30 TABLE 9: Presence or absence of variants identified in table 4 in 7 non-FHBV and 15 FHBV sequences, all of genotype D.

5 TABLE 10: Clinical data of FHBV and chronic control cases.

M= male, F= female, N/A= not applicable, n/D= not done, +ve= positive, -ve= negative, ++= highly positive, IV = intravenous needle

FHBV= fulminant HBV, CHBV= chronic HBV, dot/blot= DNA was detected by dot-blot hybridisation, ?= not known. * =Titre indicates final dilution at which sample remained positive.

10 TABLE 11: Primers used for PCR

Table 11 shows the sequences of primers in 5' to 3' direction used for PCR. The underlined sequences are the restriction sites. Outer= primers used for 1st round PCR, Inner= primers used for nested PCR.

TABLE 12: Sense strand oligonucleotides used for gel shift.

15 Table 12 shows the oligonucleotide sequences used for gel shift analysis. Only sense strands are shown here. Nucleotide positions of 5' and 3' ends, numbered from the Eco RI site, are given above oligonucleotide sequences.

TABLE 13: Luciferase value correlated with variation in the CURS, BCP and pre core region.

20 Variants found in the region between 1549-1974, which contains the CURS and the BCP/Enh-II complex, are considered. The clustering pattern and patie number follows that in the previous study (Bollyky *et al*, 1997, submitted). CURS = core upstream regulatory sequence, BCP= basal core promoter. Enh-I Enhancer II. CHBV-1 and -2 are infective contacts of fulminant cases. H= HIGH (luciferase value > 7), I= intermediate (luciferase value 2.0-7.0), N/A= No applicable, N=Normal (luciferase value < 2), Δ= deletion, ₁₈₃₈A₁₈₃₉ and ₁₈₃₈AC₁₈₃₉ denote an insertion of A or AC between the noted nucleotide positions. The standard deviation is derived from at least 6 replicates for each

25 construct. Rapid/slow disease progression was a clinical judgement; allocations to categories were made before these studies were undertaken. Underlined variants are not unique but significant in comination with another variant(s).

DISCUSSION

We report the clustering of FHBV sequences from epidemiologically unlinked sequences within particular viral lineages. Further, these viral cluster relationships seemed to predict death or survival in the absence of additional confounding factors for poor prognosis such as pregnancy, coinfection with HDV and older age. This is conclusive evidence of a viral genetic basis to FHBV.

Further, 21 of the 26 X gene FHBV sequences had a combination of variants in the complex which regulates pregenomic and precore production (the NRE, the BCP region of enhancer II and the A₁₈₉₆ precore variant), plus one of 3 alterations which might be expected to affect the production or function of X gene protein products (aberrant methionine or cysteine residues or enhancer I variants). These combinations, or motifs, were nearly exclusive to FHBV sequences. We propose a novel concept in studies of HBV: individually, many of these variants are not unique to FHBV sequences but that their pathogenic significance lies in their interaction.

We hypothesise that these motifs are associated with common functional effects. The significantly higher rates of nucleotide evolution in the C and X genes and in the HBV genome as a whole (results to be published separately) suggest that an increased rate of evolution is a hallmark of FHBV. The elevated rate of synonymous changes in X and of evolution in non-antigenic regions in C are consistent with this interpretation. Further, we have shown that the BCP region of A₁₈₉₆-associated FHBV sequences has significantly increased transcription efficiency (Yasmin et al., submitted). This supports one report of increased replication and encapsidation efficiency shown for an individual FHBV sequence⁵. Another novel, though related, concept to arise from this work is that specific variants can be linked to a different clinical outcome if they occur outside of their usual genotype context. Further, particular genotypes may predispose patients to FHBV: 15 of the 26 X gene FHBV sequences were of genotype D (out of 35 sequences available for that genotype).

These motifs, however, are clearly not entirely equivalent; the particular variants which characterise individual clusters of FHBV sequences appear to result in their distinct clinical features. A fatal outcome was associated with every FHBV sequence with either the T₁₇₆₂ or A₁₇₆₄ variants. In two other studies, 3 of 4¹⁸ and 4 of 5¹⁷ such cases were fatal. In a recent computer modelling study, these variants have been suggested to lead to alterations in RNA superstructure encompassing the encapsidation signal loop from which DNA replication is initiated⁴² and are part of the TATA complex involved in initiation of precore and pregenomic transcripts⁴³. Along with positions 1762 and 1764, position 1773 is part of the TATA complex. Every FHBV case associated with aberrant C₁₇₇₃ was similarly fatal. Different classes of FHBV are also associated with distinct selective forces,

5 as the A₁₈₉₆ FHBV sequences are shown here to have different patterns of evolution in the C gene than FHBV sequences without this variant.

The A₁₈₉₆ variant has been found in both CHBV as well as FHVB cases and it has previously suggested that the transmission of the A₁₈₉₆ variant and HBeAg negative status results in FHBV pathogenesis^{5,6,10}. Building on our recent report of the transmission of the A₁₈₉₆ variant, and
10 presumably HBeAg negative status, on a significant scale (Bollyky, et al. submitted for publication), here we conclusively show that the combination of A₁₈₉₆ with other FHBV associated variants distinguishes A₁₈₉₆ chronic from A₁₈₉₆ fulminant cases. Lending further support to this hypothesis of non-equivalent effects of A₁₈₉₆ in FHBV and CHBV cases, we have also shown here that A₁₈₉₆ FHBV sequences have different patterns of evolutionary change than A₁₈₉₆ CHBV sequences.

15 The phylogenetic clustering of unlinked FHBV cases, when FHBV is itself a viral transmission dead end, would suggest that certain viral genetic contexts, such as those defined by strain and genotype identity, might either be particularly susceptible to acquiring specific FHBV associated variants or already possess some elements of the FHBV motif combinations. We propose that the symptomless contact who transmits HBV resulting in fulminant hepatitis has a heterogeneous
20 population of HBV strains, or quasispecies characteristic of quickly evolving viruses⁴⁴, some of which possess all the necessary elements required to cause FHBV in *de novo* infections by triggering a massive immune response in the presence of elevated rates of viral replication.

It is clear that looking for a single mutational cause for all cases of FHBV is too simplistic. The results presented here, based on a large dataset and the application of phylogenetic and statistical
25 methods, conclusively show that there are multiple sequence routes to FHBV pathogenesis which we believe share common functional features. While we do not discount that transmission related effects, co-infection with other viruses, and host factors such as HLA, as shown to be related to viral clearance⁴⁵, may be involved, we believe that viral sequence is a necessary prerequisite and central to the causation of fulminant hepatitis B. Greater consideration must certainly be given to the
30 interactions of multiple viral factors and the context within which specific variants occur. Closer examination of the multiple routes leading to FHBV promises to shed light on HBV immunomodulation and transcriptional and translational control.

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5 **FIGURES 1 to 6**

Figure 1

10 Organisation of genes and regulatory regions along the HBV genome. CURS = core upstream regulatory region. BCP = basal core promoter. NRE = negative regulatory element. Pre-C = pre-core gene. C = core gene. X = X gene. DR = direct repeat (DR1 initiates reverse transcription, DR2 second strand synthesis). The DNA is separated into two strands for clarity (though both strands are the coding strand). The ruler indicates nucleotide positions numbered from the unique EcoR1 site.

Figure 2

15 Clustering of FHBV sequences on the maximum likelihood tree of the X gene. For the 191 C gene sequences the Ts/Tv was determined to be 1.24 with the relative rates of evolution as 0.87:0.62:1.51 at codon positions 1, 2 and 3, respectively. Lineages which possess FHBV status are indicated. Horizontal branch lengths are drawn to scale. Clusters of sequences, as defined as FHBV lineages which have no intervening non-FHBV sequences, are numbered in bold. Genotype of each major branch is given. Genotype E is indistinguishable from genotype D within the X gene, as has been noted previously 19.

20 Figure 3

Clustering of FHBV sequences on the maximum likelihood tree of the C gene. For the 114 X gene sequences the Ts/Tv ratio and the codon-specific relative rates of evolution were found to be 1.28 and 1.49:1.04:0.50 at codon positions 1, 2 and 3, respectively. Lineages which possess FHBV status are indicated. Horizontal branch lengths are drawn to scale. Clusters of sequences, as defined as FHBV lineages which have no intervening non-FHBV sequences, are numbered in bold. Genotype of each major branch is given.

25

Figure 4

A relative rate test compares rates of evolution in two phylogenetically related viruses, *a* and *b*, by comparing their DNA and amino acid sequences to that of a third, reference virus *c*, which is more distantly related to both (i.e. it is an outgroup). Because *a* and *b* share more recent common ancestry with respect to sequence *c*, they should be equally distant to this outgroup sequence if they are evolving at the same rate.

30

5 Figure 5

The complete DNA sequence data from 1000 to 2500 for seven non-FHBV patients (comparison) and fifteen FHBV patients (invention), all genotype D, showing the mutations from the normal at various variant positions.

Figure 6

10 Diagrammatic representation of various mutational motifs associated with FHBV.

SECTION II

Here we show that the *cis*-acting sequences from four of the clusters have increased transcriptional activity *in vitro* and that the variant promoter sequences have lost nuclear factor binding activity.

15 **PATIENTS AND METHODS***Cases and controls*

In the phylogenetic analysis (Section I), 16 FHB cases, 3 FHB contact cases and 2 AHB cases were studied. In the functional analysis reported here, we chose 11 FHB or FHB contact cases from that study and 7 additional chronic hepatitis (CHB) controls not associated with FHB following transmission. Clinical and demographic details are given in Table 10. We grouped contacts with FHB having shown previously that their HBV sequences cluster phylogenetically. Figure 7 gives the sequences of all the subjects from the beginning of the core upstream regulatory sequence (CURS) to the end of enhancer II (nt 1643- 1849) indicating the variants we defined previously as being more common in FHB than in chronic cases, taking into account the genotype-specific variability. We have included two additional laboratory standard control sequences of subtypes *adw2* (genotype A) and *ayw* (genotype D).

DNA extraction, PCR, sequencing and sequence analysis

In brief, 25 ul serum was digested overnight in 1mg/ml proteinase K at 37° C. DNA was extracted once each with phenol/chloroform (1:1), once with chloroform and precipitated in ethanol. Finally, the DNA was resuspended in 15 ul dH₂O. For PCR amplification, 5 ul extracted DNA was amplified by 25 pmol each of sense and antisense outer primers. 1 ul of first round product was nested, or hemi-nested. A list of primers is given in Table 11. DNA was sequenced directly using

5 Sequenase version 2.0 (USB, Amersham, UK).

Luciferase assay

Constructs were based on vector pBL (23), which includes the luciferase gene but without its own complete promoter. Nucleotides 1549-1975 of HBV (generated by PCR using primers C8m & BC3, Table 11), which includes the negative regulatory elements (NRE), CURS, BCP, enhancer II (EnhII) and a portion of core gene were cloned into pBL upstream of the luciferase gene. Clones were sequenced to verify their identity. Luciferase assays were performed as previously described (24). In brief, 10ug plasmid DNA was transfected into two 60 mm plates containing 50-70% confluent Huh7 hepatocyte cells by standard calcium phosphate transfection. To overcome the effect of variable transfection efficiency, each plate was co-transfected with 0.2 ug pSV vector containing the β -galactosidase gene. 72 hrs after transfection, cells were lysed with 25mM Tris phosphate, 10% glycerol and 0.1% triton X-100. 50 ul of cell lysate was mixed with 350 ul of assay buffer (20mM tricine, 1.07mM $\text{MgCO}_3 \cdot 5\text{H}_2\text{O}$, 2.67mM MgSO_4 , 0.1mM EDTA, 33.3mM DTT, 270mM Co-enzyme A, 470mM luciferin, 530mM ATP. pH 7.8), and luciferase production was either measured in an automated luminometer, or with the Luciferase assay kit (Promega, Madison, USA) following the manufacturer's instructions. For each construct, the assay was done at least 3 times; for each assay, duplicate plates were used; and for each plate, duplicate measurements were taken. The β -galactosidase assay was performed with the same cell extract. 50 ul of cell extract was incubated with a buffer (50mM Na_2HPO_4 , 39mM NaH_2PO_4 , 1mM HCl, 1mM MgSO_4 , 1mg/ml ONPG) at 37°C until a pale yellow colour developed. The OD at 405 nm was measured. The luciferase value for each experiment was calculated by dividing the mean unadjusted luciferase value by the mean β -gal value and the luciferase value for each construct was the mean of all experiments.

Preparation of nuclear extract and mobility shift analyses

Nuclear extracts were made from HeLa cells and HuH7 cells by a method described by Trautwein et al (25), and stored at -70° until used; for each experiment, a fresh aliquot was used. Complementary oligonucleotide pairs corresponding to the variant HBV sequences in Enh-I and Enh-II/BCP complex (Table 3) were made either using an in-house oligo-synthesizer or purchased from Oligold (Eurogentec, Seraing, Belgium). Oligonucleotides were annealed to make double-stranded DNA was labelled with ^{32}P γ ATP. Labelled oligonucleotide was purified from unincorporated ^{32}P with Sephadex G-50 spin column (Pharmacia, Uppsala, Sweden). For gel shift experiments, 3-5 x 10⁵ cpm

5 of labelled oligonucleotides were mixed with 4 ul of binding buffer (final concentration: 25mM HEPES pH 7.6, 5mM MgCl₂ and 34mM KCl), 1µg poly dIdC, 2µg BSA, 2 ul proteinase inhibitor cocktail (20mM DTT, 2mM PMSF) in a total of 10 ul volume and kept on ice. The mix was then added to equal volume of dialysis buffer (25mM Hepes, 1mM EDTA, 40mM KCl, 11.4% glycerine) containing 0, 1 and 3 ug of nuclear extract. The mix was left at room temperature for 15-20 mins
10 before analysis on a non-denaturing 6% polyacrylamide gel.

HBV core protein distribution in vitro

To express the core gene with its homologous promoter, nts 1549-2458 were generated by PCR using C9y & C2y as outer and C8y & C4y as inner primers (Table 11), then cloned into pT7-blue (Novagen, AMS Biotechnology, Oxon, UK). The *PstI-XhoI* fragment was recloned into vector
15 pKLt55 (a generous gift from Dr Walter, Genetics Dept, Glasgow University) whose own promoter had been removed. To study the effects of core protein sequence variability on intracellular distribution without any effects from its homologous promoter, a fragment of nt 1818-2458 containing core gene only, was generated by PCR from the pKLt55 construct using primers C5e and C4h and cloned into vector pRK5 (a generous gift from Prof H Will, Hamburg, Germany) which
20 contains the SV40 origin of replication and the CMV early promoter. Core constructs under the control of the homologous promoter were expressed in HepG2 hepatocyte cells and those under the heterologous promoter were expressed in both Cos7 and HepG2 cells. Briefly, 5ug plasmid DNA was transfected into cells on a 16mm coverslip in 35mm dish by standard calcium phosphate precipitation method. Cells were harvested 48-72 hrs after transfection, fixed with paraformaldehyde, labelled with
25 polyclonal anti-core IgG (Sigma Chemical Company, St Louis, USA) as 1° antibody and anti-rabbit mouse IgG as 2° antibody. Cells were examined using a Nikon Microphot-SA fluorescence microscope.

RESULTS

Fulminant hepatitis B virus constructs have a higher transcription level

30 A sequence greater than 400 nt containing the NRE, CURS, BCP, Enh II and the 5' end of the core gene from a total of 20 patients was cloned into a luciferase-expressing vector. Eleven of 20 were FHB; 4 of them were HBeAg negative in the first available samples. Two were laboratory standard *adw* (HBV *adw2* in Genbank; genotype A) and *ayw* (Xxhepav in Genbank; genotype D) subtype controls (both HBeAg producing) and the remaining 7 were CHB controls with well known

clinical characteristics. Table 13 shows the luciferase level correlated with variation in the *cis* acting and pre-core regions. Seven of 11 FHB cases, all with variation in the first two AT rich regions of the BCP (Figure 7) showed substantially higher luciferase activity compared to control *adw* which we considered as background or normal. Six of these 7 cases also had A₁₈₉₆ and one with G₁₈₉₆ sequence had an A insertion at 1838 and was therefore an HBeAg negative strain. Two of 11 cases (FHBV-8 and FHBV-9) had an intermediate level of luciferase level compared to control *adw*. For case FHBV-8, 2 clones were tested, one with A₁₈₉₆ and the other with G₁₈₉₆, but both had variant BCP regions. Both clones showed the same level of luciferase activity, but this was not consistent (higher standard deviation). Case FHBV-9 was G₁₈₉₆ and invariant in the BCP, but had a single variant in the CURS. The last 2 of the 11 FHB cases (FHBV-12 and FHBV-16) had normal luciferase activity, and contained G₁₈₉₆ with almost identical variation in the CURS and BCP. Only one of these variants was in the third AT rich region and both sequences were invariant in the first two AT rich regions. Six of 7 control CHB cases showed normal luciferase production comparable to *adw* control. Three of 7 CHB controls had HBeAg-producing sequences (G₁₈₉₆), the remainder were A₁₈₉₆.

Turning to specific variants, of those in the BCP, T₁₇₆₂ (in three) and A₁₇₆₄ (in five) were the most common in CHB (5 of 7 cases). These variants also were common in FHB associated with high luciferase expression but always were accompanied by T₁₇₆₆ and/or A₁₇₆₈. This indicates that T₁₇₆₂ and A₁₇₆₄ alone are not sufficient to affect transcription in our system. However, T₁₇₆₂ (without A₁₇₆₄) with one other unique variant (A₁₈₂₆) appeared to be crucial in case FHBV-1. The only CHB control case which had intermediate luciferase activity (I-40), was HBeAg positive and contained a deletion in the BCP from nt1754-1762 (Figure 7). The functional effect of combinations of non-unique variants was further seen in the linkage of variants at nts 1727 and 1740. G₁₇₂₇ and T₁₇₄₀ together were observed 4 times, but only in the group of 7 high luciferase-producing FHB sequences. The control *ayw* subtype had higher luciferase activity compared to *adw* and had considerable differences in the BCP/enhancer II complex, particularly at positions 1678, 1727, 1740 and 1773 (Figure 7). Con-*ayw* had A₁₇₂₇ and C₁₇₄₀; a number of FHB and control CHB cases also contained A₁₇₂₇, but always with T₁₇₄₀. This is a remarkable parallel with our finding of the 4 FHB cases with G₁₇₂₇ and T₁₇₄₀. T₁₇₇₃ was found in 8/11 of the FHB cases with high or intermediate luciferase activity. Three FHB cases had T₁₆₇₈, 2 of them had high and 1 intermediate luciferase activity; con-*ayw*, the control with intermediate activity was the only control sequence to have T₁₆₇₈. In summary, although the BCP is generally variable, some unique variants, but more importantly, combinations of variants ("motifs"), were associated with high or intermediate transcriptional activity, nearly always in FHB cases.

5 *Luciferase activity correlates with disease progression*

There was a correlation between high luciferase expression, rapidity of disease progression, and seroconversion to anti-HBe. All 7 FHB cases with high luciferase expressing sequences had rapid disease progression regardless of their clinical outcomes (Tables 1 and 4). In contrast, FHB cases with intermediate or normal luciferase expression had slow disease progression. There was a
 10 correlation between high luciferase expression and HBeAg status in FHB cases. Six of 7 FHB cases with high luciferase expression were HBeAg negative on admission and seroconverted to anti-HBe, other 4 FHB cases with intermediate and normal luciferase expression were HBeAg positive on admission and 1 of them seroconverted to anti-HBe. In contrast, Four of 7 HBeAg negative CHB cases had normal luciferase expression.

15 *BCP and enhancer 1 sequences from FHB have different nuclear factor binding patterns to non-FHB controls*

To investigate the effect of BCP variation on binding of transcription factors derived from nuclear extracts, complementary oligonucleotides spanning nts 1748-1783 from 6 FHB cases with variability in the BCP and from *adw* subtype as a non-FHB control were synthesised. As can be seen from
 20 Figure 7, the chosen oligonucleotides included most of the variants previously identified as linked to FHB. Figure 8 gives the results of nuclear factor binding and Figure 10 compares the sequence to cartoons of the banding patterns. The non-FHB sequence (OL-*adw*) shows 3 clear complexes (Figure 8a), whereas all FHB sequences except one (FHBV-1), which had only one mutation (T₁₇₆₂), bound poorly, or not at all, with complexes II and III. Case FHBV-1 (Figure 8c) which showed a
 25 similar binding pattern compared to non-FHB control (OL-*adw*), had two bands in complex-II. In order to identify liver specific complexes, nuclear extracts from HeLa cells were made, and the binding assay repeated using oligonucleotides from 3 FHB and 1 control non-FHB cases. From Figure 9b, it is clear that complexes II and III are not hepatocyte specific. The BCP variant oligonucleotides showed a similar pattern of binding to both nuclear extracts (Figure 9b, lane 3, 4 and
 30 5). A competitive experiment using unlabelled oligonucleotides as a cold competitor showed the specificity of the interactions (Figure 8.d).

A number of FHB sequences had variants in Enh-I, particularly T₁₀₅₀C, G₁₂₄₉C and T₁₂₅₀. Four pairs of oligonucleotides with or without Enh-I variants were synthesised (Table 12) and bound to nuclear extracts of HuH7 cells. Figure 11 shows that variant G₁₀₅₀ (oligonucleotide em-1) does not
 35 have any effect on the nuclear factor binding pattern, but variants T₁₂₄₉ and C₁₂₅₀ (oligonucleotide em-

2), in contrast to G₁₂₄₉ and T₁₂₅₀ (oligonucleotide ew-2), do not give rise to complex 3.

Finally, a nuclear factor binding assay was performed using an oligonucleotide with an insertion of A at 1838 (Table 12) as detected in two patients (CHBV-1 and FHBV-15). No difference was observed in the binding pattern (data not shown).

Expression and distribution of core protein was not different in FHBV.

To investigate the pattern of core protein and its intracellular distribution under the control of the homologous promoter, an area spanning nt 1549-2458 from 13 patients (8 FHB or contacts of FHB, 4 CHB and 1 AHB) was cloned into vector pKLT55. Core proteins were expressed in HepG2 cells and labelled with polyclonal anti-core rabbit IgG. The influence of core protein sequence on intracellular distribution was addressed using core nt 1818-2458 from 11 FHB, 4 CHB and 1 AHB cases in vector pRK5 under control of the CMV early promoter. The core proteins were expressed in both COS7 and HepG2 cells. HBcAg distribution was observed in both cytoplasm and nucleus and there was no obvious difference in the level of HBcAg expressed in FHB and control cases. A similar level of expression was observed using either homologous or heterologous promoters (data not shown).

DISCUSSION

A *cis*-acting transcriptional regulatory element of approximately 100 bp (nt 1743-1849) has been shown to function as the BCP (26). This region also contains the 3' end of the X gene and the 5' end of the encapsidation signal and is sufficient for accurate initiation of both pre core and pregenomic RNAs. A sequence element upstream of the BCP, the CURS, binds to hepatocyte nuclear factors HNF-3, HNF-4, C/EBP or other transcription factors, and stimulates the activity of the BCP (27,28,29). Enh-II overlaps the BCP and has a stimulatory effect on the BCP, as well as the SP-I and SP-II promoters (28,29). Three AT rich regions have been mapped within the BCP region, the first two of which serve as the initiation sites for two longer pre-core, and the third one a shorter pregenomic, mRNAs (30). The two most common variants in these AT rich regions are T₁₇₆₂ and A₁₇₆₄, observed in association with both chronic HBV carriers and FHB cases. Initially they were believed to be correlated with HBeAg negative phenotype, perhaps acting by downregulating precore mRNA synthesis (30,31). However, this has not been confirmed by others (32), although there may be an association with lower levels of HBeAg (32).

One hypothesis to explain the massive liver cell injury in FHB is that altered binding of

transcription factors leads to increased virus replication which, in predisposed individuals, induces an exaggerated immune response. We have shown clearly that *cis*-acting regions from four phylogenetically linked clusters of FHB viral sequences which are epidemiologically unrelated have enhanced transcriptional activity *in vitro*. Further, this effect was confined to those FHB-associated sequences containing variants in BCP as well as A₁₈₉₆. The only exception to this rule was CHBV-1, a symptomless male contact implicated in causing FHB in successive wives (4). His sequence also showed high luciferase expression despite G₁₈₉₆ but this was accompanied by an A insertion at nt1838 and C₁₈₆₂ which lies within the encapsidation signal and might have altered the RNA secondary structure. FHB cases with G₁₈₉₆ sequences (clusters 3 and 7) had normal or intermediate luciferase levels. Two of these cases with normal transcription activity had variants in the BCP, one of which was in the third AT rich region (at nt 1794); its effect on transcription is thus unknown, but is unlikely to be important. It is illuminating that A₁₈₉₆ sequences from chronic carriers not associated with FHB cases had normal transcriptional activity whilst A₁₈₉₆ containing contacts of FHB cases had levels similar to A₁₈₉₆-FHB cases. A₁₈₉₆ containing chronic carrier controls also had variants in the BCP, commonly T₁₇₆₂ and A₁₇₆₄. But in FHB cases these two variants were accompanied by T₁₇₆₆, A₁₇₆₈ or both. While this manuscript was in preparation, these latter two variants were found based on sequence generated from a single patient, to be associated with an elevated rate of replication (33). It is clear that the two most common variants in the BCP (T₁₇₆₂ and A₁₇₆₄) do not contribute to the level of transcription unless they occur in combination with other variants. This substantiates the conclusions from the phylogenetic analysis that there are multiple sequence motifs in FHB-associated cases which have a common functional outcome and that A₁₈₉₆ itself is not, *per se*, the major factor but is a marker for other variation within the *cis* acting region of the genomes associated with it. Clearly, this helps to explain why there are large numbers of persons infected with A₁₈₉₆ strains but very few cases of FHB. As FHB as a clinical entity is rarely, if ever, transmitted, it must also be true that this increased transcriptional activity must be suppressed or otherwise offset in chronic carriers who have it, as it was found that core promoter variation in chronic patients has no effect on transcription (34).

The second finding of this work is that, in most cases with increased transcription, binding of one or more nuclear factors is impaired. Oligonucleotides were synthesised which encompass the regions in BCP which bind several transcriptional regulatory proteins including a liver specific factor C/EBP (29). A recent study found that T₁₇₆₂ and A₁₇₆₄ inhibition of binding of nuclear factors (35) is liver specific, as nuclear extracts from HeLa cells led to fewer bands. In our study, the missing bands were

5 not liver specific, as nuclear extract from HeLa cells showed the same pattern of binding. Figure 11, which schematically correlates the variation in the BCP with nuclear factor binding patterns, indicates that particular nuclear protein binding patterns are associated with BCP variants. The oligonucleotide containing only one variant (FHBV-1) showed a similar pattern of binding to the non-FHB control, indicating that T₁₇₆₂ alone is unlikely to inhibit binding of nuclear factors. However, the
10 oligonucleotide derived from case FHBV-15, which contained one unique variant (C₁₇₅₂) and one variant out of its usual genotype context (A₁₇₅₇) showed binding inhibition. Oligonucleotides from other FHBV cases with variants in the BCP showed a similar pattern of inhibition. This indicates that a number of combinations can lead to lack of binding to transcription factors to a similar functional outcome. All of these variant cases are associated with high or intermediate luciferase activity; we
15 therefore conclude that these two complexes have an inhibitory effect on transcription.

Thirdly, we could not distinguish any differences in HBcAg distribution in liver-derived cells in culture from FHB compared to sequences from chronic carriers. HBcAg was seen in both nucleus and cytoplasm. The amount of HBcAg production was qualitatively similar whether homologous or heterologous promoter systems were employed. In parallel studies (Dornan et al, submitted) in
20 chronic cases, sequential samples showed shifts in distribution of HBcAg from nucleus to cytoplasm. Clearly, if this is of relevance to the pathogenesis of chronic hepatitis, a different mechanism is operating in fulminant hepatitis.

Interestingly, there was a correlation between clinical parameters and transcriptional activity. High transcriptional activities were found in "rapid FHB" and their symptomless contacts. Rapid FHB is
25 characterized by undetectable viral antigens with rapid seroconversion to anti-HBe and decline into coma within two weeks of first symptoms and similarly rapid spontaneous recovery. Both symptomless contacts studied here were implicated in rapidly progressive FHB in two successive wives each (4). Liver cell necrosis and ensuing rapid clinical deterioration is explained by triggering a massive immune response which, ultimately, favours clearance of virus. Similarly, early, complete
30 cessation of virus replication should favour liver regeneration and explain the rapid clinical recovery and, ultimately, good prognosis associated with this subgroup of patients with FHB. Clearly, high levels of viraemia are not invariable with high luciferase activities because serum HBV DNA levels typically were low in such contacts and undetectable in these FHB cases except within the first week and only by nested PCR. Also, massive liver injury is not inevitable with high transcriptional activity
35 as both contacts had symptomless mild chronic hepatitis. In contrast, those patients with "slow FHB" typically show a more protracted clinical and serological course, over several weeks, and have lower

- 5 transcriptional activities than "rapid FHB". Early complete cessation of virus replication is lacking; seroconversion may be delayed as HBV DNA levels remain detectable for several weeks from first symptoms.

FIGURES 7 to 11

- 10 Figure 7: Sequences from nt 1549 to 1974 were cloned into the vector pBL. Here the sequence of CURS, BCP and Enh-II is only shown. Patient numbers were followed as for previous study in Section I. CHBV-1 and -2 are contacts of FHB cases. All I-numbers denote chronic carrier controls. BCP=basal core promoter CURS=core upstream regulatory sequence V=insertion X=deletion.

- 15 Figure 8: Nuclear extracts were prepared from HuH7 cells. 0.1 and 3 ug of nuclear extracts were mixed with 30,000 cpm 32 p labelled oligonucleotides(oligos) and run on 6% polyacrylamide nondenaturing gel. Figure 8A nuclear factors binding pattern of oligos from adw and CHBV-2. 3.B and 3.C nuclear factor binding pattern of 5 more variant oligos. E.D 30,000 cpm 32 p labelled oligonucleotide from FHBV-14 was mixed with 1x, 5x and 25x molar excess of unlabelled oligonucleotide as cold competitor (lanes 3, 4 and 5), added to 3 ug nuclear extract and resolved on 6% polyacrylamide gel, lane 1=no nuclear extract, lane 2= no cold competitor.

- 20 Figure 9: Nuclear extracts were made from HuH7 cells and HeLa cells. 0.1 and 3 ug of nuclear extracts were mixed with 30,000 cpm 32 p labelled oligonucleotide and resolved on 6% polyacrylamide non denaturing gel. Figure 9a shows binding of oligonucleotide derived from con-adw (OL-adw). CHBV-2, FHBV-14 and FHBV-4 with nuclear extracts from HuH7. Figure 9b shows same oligonucleotides binding with nuclear extract made from HeLa cells.

- 25 Figure 10: Cartoon representation of nuclear factor binding assay showing luciferase values and variants in the BCP; and

Figure 11: Oligonucleotides were made from normal C₁₀₅₀(OL ew-1), variant G₁₀₅₀ (OL em-1), normal C₁₂₄₉, T₁₂₅₀ (OL ew-2) and variant T₁₂₄₉, C₁₂₅₀ (OL em-2). Oligonucleotides were mixed with 0.1 and 3 ug of nuclear extracts from HuH7 cells and run on 6% polyacrylamide non-denaturing gel.

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Table 1

Cluster number Sequence number & name	Genes Genotype sequences available	Age/Sex	HbeAg	Anti-HBe	Disease type & course (rapid/slow)**	Outcome**	Country of origin of infection & comments	Reference
CLUSTERED AND RELATED SEQUENCES								
1								
1.FHBV1	C,X	25/F	-	-	FHBV-Rapid	Died [@]	UK	\$
2.FHBV2	C,X	23/F	-	+	FHBV-Rapid	Survived	UK	\$
3.FHBV3	C,X	17/F	-	+	FHBV-Rapid	Survived	UK	\$
4.FHBV4	C,X	18/F	-	+	FHBV-Rapid	Survived	UK	\$
5.FHBV5	C,X	19/M	+	-	FHBV-Rapid	Survived	UK	\$
6.FHBV6	C,X	23/F	-	-	FHBV-Rapid	Survived	UK	\$
7.AHBV1	C,X	NA	+	-	AHBV-NA	Survived	UK	\$
8.FHBV7	C,X	21/F	-	-	FHBV-Slow	Survived ^{#@}	contact of FHBV6 Italy	\$
2								
9.FHBV8	C,X	25/F	-	+	FHBV-Rapid	Died	UK	\$
10.CHBV1	C,X	35/M	-	+	CHBV-Rapid	Died	UK, fatally infected two successive wives	6,\$
3								
11.FHBV9	C,X	23/M	+	-	FHBV-Slow	Died	UK	\$
12.FHBV10	C,X	30/F	+	-	FHBV-Slow	Died	UK	\$
13.FHBV11	C	45/F	+	-	FHBV-Rapid	Died	Nepal	\$

14.HBVP3CSX	C,X	D	F	-	NA	FHBV-NA	Survived*	Greece	21,24
15.HBVP2CSX	C,X	D	M	NA	NA	FHBV-NA	Died	Greece	24
16.*HBVP1PC	C	D	F	-	-	CHBV-NA	Survived*	Greece, contact of HBVP3CSX	21
17.*HBVP2PC	C	D	M	-	+	CHBV-NA	Survived*	Greece, contact of HBVP1PC	21
<u>5</u> 18.FHBV12	C,X	D	20/F	+	-	FHBV-Slow	Died ^p	Spain	\$
19.FHBV13	C,X	D	25/M	-	-	FHBV-Slow	Died ^p	Swiss	\$
<u>6</u> 20.*FHBV14	C,X	D	29/F	-	+	FHBV-Rapid	Died [@]	Pakistan	\$
21.CHBV2	C,X	D	35/M	-	+	CHBV-Rapid	Died	UK, fatally infected two successive wives	6,\$
22.HPBMUT	C,X	D	NA	-	+	FHBV-Rapid	Died	Israel, one of 5 fatally infected in an outbreak attributable to a single source	35
23.*HPBC5HK02	C,X	C	40/F	-	+	CHBV-NA	Died	Japanese, contact of one case	4,25
24.*HPBETNC	C,X	C	infant	-	-	CHBV-NA	Died	Japanese, contact of two cases	7,26
25.HBVP4PCXX	C,X	D/A ^s	M	-	NA	FHBV-NA	Died	Greece	21
26.HBVP5PCXX	C,X	D/A ^s	F	-	+	CHBV-NA	Died	Greece, contact of HBVP4PCXX	21
<u>INDIVIDUAL UNLINKED CASES</u>									
27.HBVP4CSX	C,X	B	M	-	NA	FHBV-NA	Survived	Chinese	24
28.FHBV15	C	D	27/M	-	+	FHBV-Slow	Survived*	USA	\$
29.FHBV16	C,X	A	30/M	+	-	FHBV-Slow	Died	Cameroon	\$
30.HPBC4HST2	C,X	C	57/M	-	+	FHBV-Slow	died	Japanese	25

Table 2.

Nucleotide Sequence*

Region

Full sequence	0.024
1373-1631	0.906
(CURS)1631-1742	0.678
(BCP)1742-1838	0.009

Amino Acid Sequence

Full sequence	0.050
1-86	0.255
(CURS)87-123	0.636
(BCP)124-154	0.042

Nucleotide Sequence

Region	FHBV	G ₁₈₉₆ FHBV	A ₁₈₉₆ FHBV	A ₁₈₉₆ non-FHBV
Full sequence (1901-2458)	0.045	0.022	0.009	0.018
Region 1 (1963-2020)	0.154	0.787	0.076	0.715
CD4 epitope (2050-2107)	0.610	0.361	0.944	0.036
Anti-HBc/e1 (2122-2161)	0.919	0.281	0.141	0.036
Anti-HBc/e2 (2200-2251)	0.724	0.281	0.014	0.281
Anti-HBc/e3 (2290-2305)	0.787	1.000	0.022	0.181
Antigenic regions	0.108	0.052	0.009	0.014
Non-antigenic regions	0.039	0.043	0.013	0.051

Amino Acid Sequence

Region	FHBV	G ₁₈₉₆ FHBV	A ₁₈₉₆ FHBV	A ₁₈₉₆ non-FHBV
Full sequence	0.108	0.151	0.013	0.013
Antigenic regions	0.162	0.295	0.009	0.014
Non-antigenic regions	0.235	0.052	0.024	0.183

Table 4.

	Enhancer I	NRE	BCP/Enhancer II*	Pre-C	Aberrant Cysteines	aberrant Methionines
Nucleotide variant	G ₁₀₅₀ T ₁₁₁₅ T ₁₂₄₉ C ₁₂₅₀	1633 [±]	T ₁₇₆₂ A ₁₇₆₄ T ₁₇₆₆ A ₁₇₆₈	1390 [±] T ₁₄₄₉ T ₁₅₈₇	A ₁₃₈₆ G ₁₆₃₇ G ₁₇₅₄ A ₁₇₉₄	
Translation [§]			M130 I131 Y132	6 [±] 26 72 78 [±]	5 [±] 88 127 141	
FHBV Prevalence	10/26 2/26 10/26 14/26 10/26 13/26	6/26 7/26 4/26 2/26 3/26	16/30	2/26 11/26 1/26 1/26	3/26 9/26 5/26 1/26	
Non-FHBV Prevalence	4/52 1/52 3/52 3/52 3/88 6/88	16/88 15/88 3/88 3/88 0/88 43/161	2/88 9/88 0/88 1/88	1/88 2/88 0/88 0/88		

CLUSTERED AND RELATED SEQUENCES

	1	2	3
1. FHBV1	+	-	+
2. FHBV2	+	+	+
3. FHBV3	+	+	+
4. FHBV4	+	+	+
5. FHBV5	+	+	+
6. FHBV6	+	+	+
7. AHBV1	+	+	+
8. FHBV7	+	+	+
9. FHBV8	-	+	-
10. CHBV1**	-	+	-
11. FHBV9	-	-	-
12. FHBV10	-	-	-

13.FHBV11	NA	NA	NA	NA	NA	NA	-	-	-	-	-	-	NA	NA	NA	NA	-	-
4																		
14.HBVP3CSX	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-
15.HBVP2CSX	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
16.HBVP1PC	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	NA	NA	NA	NA	NA	NA
17.HBVP2PC	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	NA	NA	NA	NA	NA	NA
5																		
18.FHBV12	+	-	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-
19.FHBV13	+	-	+	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-
6																		
20.FHBV14	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-
21.CHBV2	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-
22.HPBMUT	-	-	-	-	+	+	+	+	+	+	+	+	-	+	-	-	-	-
23.HPBC5HK02	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	-	-	-
24.HPBETNC	-	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	-	-
25.HBVP4PCXX	-	-	-	+	-	-	+	-	-	-	+	+	-	-	+	-	-	-
26.HBVPCCXX	-	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+	-	-

INDIVIDUAL UNLINKED CASES

[illegible]

TABLE 5

40

	Enh-I	NRE 1633/1634	Enh-II/CURS/BCP
<u>FHB cases in this study</u>			
FHBV-1	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/-	T ₁₇₆₂ A ₁₈₂₆
FHBV-2	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/+	-
FHBV-3	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/-	T ₁₈₂₁
FHBV-4	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/+	G ₁₇₅₄
FHBV-5	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/+	G ₁₇₅₄
FHBV-6	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/+	G ₁₇₅₄
FHBV-7	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/+	-
FHBV-8	-	-/-	T ₁₇₆₂ A ₁₇₆₄
FHBV-9	-	-/-	C ₁₇₄₀ C ₁₇₇₃
*FHBV-10	-	-/-	-
*FHBV-11	n/a	n/a	-
FHBV12	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/+	A ₁₆₅₃ , C ₁₇₀₃ , G ₁₇₅₄
FHBV13	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/+	C ₁₇₇₃
FHBV14	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	-/-	T ₁₇₆₄ G ₁₇₆₆
FHBV15	n/a	n/a	C ₁₇₅₂ 1838 AC ₁₈₃₉
FHBV16	-	-/-	T ₁₇₀₃ T ₁₈₀₉ T ₁₈₁₂
CHBV-1	-	-/-	A ₁₇₆₄ 1838 A ₁₈₃₉
CHBV-2	G ₁₀₅₀ , C ₁₂₅₀	-/-	T ₁₇₆₂ A ₁₇₆₄ T ₁₇₆₆ A ₁₇₆₈
AHBV-1	G ₁₀₅₀ , T ₁₂₄₉ , C ₁₂₅₀	+/+	G ₁₇₅₄
<u>FHB cases from Genbank</u>			
HBVP3CSX	-	-/+	-
HBVP2CSX	-	-/+	-
HBVP1PC	n/a	n/a	n/a
HBVP2PC	n/a	n/a	n/a
HPBMUT	-	-/-	T ₁₇₆₂ , A ₁₇₆₄ , T ₁₇₆₆ , A ₁₇₆₈
HPBC5HK02	-	-/-	A ₁₇₆₄ , T ₁₇₆₈
HBVP4PCXX	-	-/-	T ₁₇₆₂ , A ₁₇₆₄
HBVP5PCXX	-	-/+	C ₁₇₇₃
HBVP4CSX	-	-/+	C ₁₇₇₃
HPBC4HST2	-	-/+	T ₁₇₆₂ , A ₁₇₆₄ , T ₁₇₆₈

TABLE 5-2

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X protein		A ₁₈₉₆	Clinical factors	Comments/ Motif (Table 6 refs)
Cys/Met	Unique			
C26 M88 M130	L151	+	Pregnant	Motifs- all
C26 C72 M88	F75	+		Motifs- 1, 2, 3, 6, 7, 8
C26 M88		-	-	Motifs- 6, 7, 8
C26 M88 M127	F6	+	-	Motifs- 1, 2, 3
C26 M88 M127	T68	-	-	Motifs- 6, 7, 8
C2, M127		-	-	Motifs- 6, 7, 8
C26 M88		-	Pregnant	Motifs- 6, 7, 8
C6 M130	Y95 D126	-	-	Motifs- 4, 5
-	-	-	-	1 unusual variant plus variant outside genotype context
-	-	-	-	-
n/a	n/a	-	-	-
C26 M127		-	-	Motifs 4, 5, 6, 7 plus HDV coinfection
C26	N88	-	HDV	Motifs 6, 7 plus HDV coinfection
-	A146, S147	+	HDV	Motif 1, 3 plus pregnant
n/a	n/a	+	Pregnant	Unique variant in BCP plus insertion
-	A146, S147	-	-	Unique variants in BCP and X protein
M130 M141	S13, L150	-	-	Motif- 5 plus insertion
M5 M130	T127	+	-	Motif- 2, 3, 5
C26 M88 M127	-	-	-	Motifs- 6, 7, 8
-	?	+	-	A ₁₈₉₆ plus variants in NRE
-	?	-	-	Variants in NRE
n/a	?	+	-	A ₁₈₉₆

TABLE 5-3

n/a	?	+	-	A ₁₈₉₆
C26, M130	?	+	-	Motifs-1, 2, 4, 5
M5,	?	+	-	Motifs-2,5
M5, M130	?	+	-	Motifs-2,5
M88	?	+	-	Motifs-2, 5 plus C ₁₇₇₃
M88	?	+	-	Motifs-2, 5 plus C ₁₇₇₃
M130	?	+	-	A ₁₈₉₆ plus variants in BCP

TABLE 6

Position	Typical variant at that position for a given genotype	Incidence of deviation from these associations	
Nucleotide		FH BV	Non-FHBV
1633	G1633 (A, F), A1633 (B, C, D)	11/26	7/88
1634	G1634 (A, B, C, F), A1634 (D)	13/26	6/88
1773 *	T1773 (A), C1773 (B, C, D, E, F)	3/26	0/88
Amino Acid			
6**	Tyrosine/Phenylalanine (A), Cysteine (B, C, D, F)	2/26	2/88
78**	Cysteine (A), Arginine/Serine (B, C, D, F)	1/26	1/88

TABLE 7-1

MOTIFS *	1	2	3	4	5	6	7	8	Total
FHBV Prevalence	4/26	8/26	12/26	11/26	14/26	4/26	8/26	8/26	21/26
Non-FHBV Prevalence	1/88	0/88	4/88	2/88	1/88	0/88	0/88	1/88	6/88
<hr/>									
A1896 Sub-Total									
1									
1. FHBV1	+	+	+	+	+	+	+	+	
2. FHBV2	-	-	+	+	+	+	+	+	
3. FHBV3	-	-	+	+	+	-	-	-	
4. FHBV4	-	-	+	+	+	+	+	+	
5. FHBV5	-	-	+	+	+	-	-	-	
6. FHBV6	-	-	+	+	+	-	-	-	
7. AHBV1	+	-	+	+	+	-	-	-	
8. FHBV7	-	+	+	+	+	-	-	-	

TABLE 7-2

2											
9. FHIV8	+	-	-	-	-	-	-	-	-	-	-
	-	+	-	-	-	-	-	-	-	-	-
10. CHIV1	-	+	-	-	-	-	-	-	-	-	-
3											
11. FHIV9	-	-	-	-	-	-	-	-	-	-	-
12. FHIV10	-	-	-	-	-	-	-	-	-	-	-
4											
14. HBVP3CSX	-	-	-	-	-	+	-	-	-	+	-
15. HBVP2CSX	-	-	-	-	-	+	-	-	-	-	-
5											
18. FHIV12	-	-	+	+	+	+	-	-	-	-	-
19. FHIV13	-	-	+	-	+	+	-	-	-	-	-
6											
20. FHIV14	-	-	-	-	-	-	-	-	-	+	-
21. CHIV2	-	+	-	-	-	-	-	+	+	+	-
22. IPBMUT	+	-	-	-	-	-	+	-	-	-	-

TABLE 7-3

23. IIPBCSHK02	-	+	-	-	-	-	+	-
24. IIPBEINC	-	+	-	-	-	-	+	-
25. IIBVP4PCXX	-	+	+	+	+	-	+	+
26. IIBVP5PCXX	-	+	+	+	+	-	+	+
<u>INDIVIDUAL CASES</u>								
27. IIBVP4PCSX	-	-	-	-	-	-	-	-
29. IIBV16	-	-	-	-	-	-	-	-
30. IIPBC411ST2	-	-	-	-	-	-	-	-

table 8

Number	Variants				Incidence				
	A	+	B	+	C	+	D	FHBV	Non-FHBV
1.	A1896		T1762		A1764			4/26	1/88
2.	T1762		A1764		T1766			3/26	1/88
3.	T1762		A1764		T1766		T1768	3/26	1/88.
4.	A1896		A1386					3/26	0/88
5.	A1896		G1637					5/26	0/88
6.	A1896		T1449					4/26	0/88
7.	A1896		G1050					3/26	1/52
8.	A1896		T1249					4/26	0/52
9.	A1896		C1250					7/26	0/52
10.	T1762		T1449					2/26	0/88
11.	T1762		A1386					1/26	0/88
12.	T1762		G1637					1/26	0/88
13.	T1764		1390 *					1/26	0/88
14.	T1764		T1449					1/26	0/88
15.	T1764		A1386					3/26	0/88
16.	T1764		A1794					1/26	0/88
17.	T1766		T1449					1/26	0/88
18.	T1766		A1386					2/26	0/88
19.	T1768		T1449					1/26	0/88
20.	T1768		A1386					1/26	0/88
21.	1773 *		T1449					1/26	0/88
22.	1773 *		A1386					3/26	0/88
23.	C1050		1633 *					10/26	1/52
24.	C1050		1634 *					8/26	0/52
25.	T1249		1633 *					9/26	1/52
26.	T1249		1634 *					7/26	0/52
27.	C1250		1633 *					10/26	0/52

TABLE 8-2

28.	C1250	1634 *	10/26	0/52
23.	G1637	1633 *	7/26	2/88
23.	G1637	1634 *	7/26	2/88
24.	G1754	1633 *	5/26	0/88
24.	G1754	1634 *	5/26	0/88

TABLE 9

Sequence Variant

Sequence	T1115	C1250	1390	T1587	1633	G1637	T1762	T1766	1773	A1896
HBVORFS	X	.
XXHEPAV
HBVGEN1	X	.
XXHEPAV
HPBHBVAA	X	X	X	X
HBVAYWMC	X
HBVDNA	X
AHBV1	X	X	X	.	X	.	X	X	X	.
FHBV1	X	X	X	.	X	.	X	X	X	X
FHBV2	X	X	X	X	X	X	.	.	X	X
FHBV3	X	.	X	.	X	X	.	.	X	.
FHBV4	X	X	X	.	X	X	.	.	X	X
FHBV5	X	X	X	X	X	X	.	.	X	.
FHBV6	X	X	X	.	X	X	.	.	X	.
FHBV7	X	X	X	.	X	X	.	.	X	.
FHBV12	X	X	X	-	X	X	.	.	X	.
FHBV13	X	X	X	.	X	X	.	.	X	.
HBVP3CSX	.	X	X	X
HBVP2CSX	.	X	X	.
CHBV2	.	.	X	.	.	.	X	X	X	X
HPBMUT	X	.	X	X	X	X
FHBV14	.	X	X	.	.	.	X	X	X	X

TABLE 10-1

WO 98/45421

Name	Gender/age	Country of origin	Days of illness before sample	HIBsAg*	HBeAg	Anti-HIBe	HIBV DNA (dot-blot)	Transmission route	Clinical features
<u>FHB cases and contacts</u>									
FHBV-1	F/25	UK	8	N/D	-ve	-ve	N/D	?	Pregnant. Died
FHBV-4	F/18	UK	6	1:64	-ve	+ve	-ve	Sexual	Survived
FHBV-5	M/19	UK	5	1:6,400	+ve	-ve	-ve	IV use	Survived
⁵⁰ FHBV-8	F/25	UK	8	1:64	-ve	+ve	N/D	Sexual	Died
FHBV-9	M/23	UK	14	1:3,200	+ve	-ve	+ve	Sexual	Died
FHBV-12	F/20	UK	14	1:800	+ve	-ve	+ve	IV use	Died
FHBV-14	F/30	Pakistan	4	1:6,400	-ve	+ve	N/D	?	Pregnant. Died
FHBV-15	M/27	USA	9	N/D	-ve	+ve	-ve	Sexual	Transplanted
FHBV-16	M/30	Cameroon	19	1:6,400	+ve	-ve	+ve	Percutaneous	Died

table 10-2

2HBV-1	M/50	UK	N/A	1:800	-ve	+ve	N/D	N/A	Contact carrier
2HBV-2	M/40	Pakistan	N/A	1:800	-ve	+ve	N/D	N/A	Contact carrier
<u>Chronic carrier controls</u>									
I-40	21/F	Italy	N/A	++	+ve	-ve	+ve	N/A	Chronic carrier
I-59	58/F	Italy	N/A	+ve	+ve	-ve	+ve	N/A	Chronic carrier
I-69	31/M	Italy	N/A	+ve	+ve	-ve	+ve	N/A	Chronic carrier
⁵¹ I-89	27/M	Italy	N/A	+ve	-ve	+ve	+ve	N/A	Chronic carrier
I-95	49/M	Italy	N/A	+ve	-ve	+ve	+ve	N/A	Chronic carrier
I-105	33/M	Italy	N/A	+ve	-ve	+ve	+ve	N/A	Chronic carrier
I-177		Italy	N/A	+ve	-ve	+ve	+ve	N/A	Chronic carrier

TABLE 11

Name	Sequences	Restriction site	Position
C8m	5'-TCGACGGATCCGTCGTGCTCTCATCTGC	<i>Bam</i> <i>HI</i>	sense
BC3	5'-GCATTCTGCAGGAAGAAGTCAGAAGGCAA	<i>Pst</i> <i>I</i>	anti-sense
C9y	5'-CGAGCTCGAGACCACGGGGCGCACCTCTTTAC	<i>Xho</i> <i>I</i>	sense, outer
C8y	5'-CGAGCTCGAGGTCTGTGCCCTTCTCATCTGCC	<i>Xho</i> <i>I</i>	sense, inner
C2y	5'-GACCTGCAGCCCCCA/CGTAAAGTTTCCC/GACCTT	<i>Pst</i> <i>I</i>	anti sense, outer
C4y	5'-GACCTGCAGCCTTATGAGTCCAAGGG/AATA	<i>Pst</i> <i>I</i>	anti sense, inner
C5e	5'-AGTCGAATTCCA/CCCTCTGCCCTAACATCTC	<i>Eco</i> <i>RI</i>	sense
C4b	5'-GGACAGAAAGCTTCCCTTATGAGTCCAAGGG/ATA	<i>Hin</i> <i>dIII</i>	anti sense

TABLE 12

OL-adw	1748	GGAGATTAGCTTAAGGCTTGTATATAGGAGCTG	1783
OL-FHBV-1		-----T-----C-----	
OL-FHBV-4		---C---A---T-A-T-A-----	
OL-FHBV-5		---C---A---T-A-T-A-----	
OL-FHBV-14		---A---A---T-G-----	
OL-FHBV-15		---C---A---C-----	
OL-CHBV-2		---C---A---T-A-T-A---C-----	
OL-EW1	1031	CACAATGTGTTATCCTGCGTTAATGCCCTTGATG	1066
OL-EW1		-----T-----T-----	
OL-EW2	1231	CGCATGCGTGAACCTTTGTGGCTCCTCTGCCGATC	1266
OL-EW2		-----TC-----	
OL-It.wt	1783	GTAGGCATTAATTGGTCTGCGCACCAAGCACCATGC	
OL-It.in		-----A-----	
		AACTTTTTCACCTCTGCCCTAA-TCAC	1842
		-----A-----	

TABLE 13

Phylogenetic Cluster	Patients and controls	Luciferase (x 10 ⁶)	Standard deviation ±	Pre-core	Variants in the Enh-II/ CURS/BCP	Variants in the Enh-II/ CURS/ BCP (found in that particular clone)	Disease progression
1	FHBV-1	11.51 (H)	3.57	A ₁₈₉₆	T ₁₇₆₂ A ₁₈₃₆	T ₁₇₆₂ A ₁₈₂₆	Rapid
	FHBV-4	8.81 (H)	1.77	A ₁₈₉₆ A ₁₈₉₉	G ₁₇₃₄	C ₁₇₅₃ T ₁₇₆₂ A ₁₇₆₄ T ₁₇₆₆ A ₁₇₆₈ T ₁₈₁₀	Rapid
	FHBV-5	10.25 (H)	2.29	A ₁₈₉₆ A ₁₈₉₉	G ₁₇₃₄	C ₁₇₅₃ T ₁₇₆₂ A ₁₇₆₄ T ₁₇₆₆ A ₁₇₆₈ T ₁₈₁₀	Rapid
2	FHBV-8.1	2.76 (I)	1.64	A ₁₈₉₆ A ₁₈₉₉	T ₁₇₆₂ A ₁₇₆₄	T ₁₆₇₈ T ₁₇₆₄ C ₁₇₆₆ T ₁₈₄₅	Slow
	FHBV-8.5	3.65 (I)	1.16	G ₁₈₉₆	N/A	G ₁₇₄₀ C ₁₇₅₃ T ₁₇₆₂	Slow
	CHBV-1	10.70 (II)	0.82	G ₁₈₉₆	A ₁₇₆₄ 1838 A ₁₈₃₉	G ₁₇₄₀ 1838 A ₁₈₃₉	Rapid
3	FHBV-9	4.06 (I)	0.58	G ₁₈₉₆	C ₁₇₄₀ C ₁₇₇₃	G ₁₇₄₀ C ₁₇₇₃	Slow
5	FHBV-12	0.60 (N)	0.30	G ₁₈₉₆	A ₁₆₅₃ C ₁₇₀₃ G ₁₇₅₄	T ₁₇₀₃ C ₁₇₉₄ T ₁₈₀₉ T ₁₈₁₂ C ₁₈₂₁	Slow
6	FHBV-14	11.39 (II)	4.71	A ₁₈₉₆ A ₁₈₉₉	T ₁₇₆₄ G ₁₇₆₆	T ₁₆₇₈ T ₁₇₆₄ G ₁₇₆₆ A ₁₈₃₄ T ₁₈₄₅	Rapid
	CHBV-2	9.68 (II)	2.46	A ₁₈₉₆ A ₁₈₉₉	T ₁₇₆₂ A ₁₇₆₄ T ₁₇₆₆ A ₁₇₆₈	C ₁₇₅₃ T ₁₇₆₂ A ₁₇₆₄ T ₁₇₆₆ A ₁₇₆₈ C ₁₇₇₁	Rapid
						T ₁₈₁₀	
Individual cases	FHBV-15	7.98 (H)	1.12	A ₁₈₉₆	C ₁₇₅₂ 1838 A ₁₈₃₉	T ₁₆₇₈ C ₁₇₅₂ 1838 A ₁₈₃₉	Rapid
	FHBV-16	1.13 (N)	0.22	G ₁₈₉₆	T ₁₇₀₃ T ₁₈₀₉ T ₁₈₁₂	T ₁₇₀₃ A ₁₇₉₄ T ₁₈₀₉ T ₁₈₁₂ C ₁₈₂₁	Slow

TABLE 13-2

Controls	I-40	4.18 (I)	1.61	G ₁₈₉₆	N/A	Δ1754-1762 A ₁₇₃₂ T ₁₇₃₃ A ₁₇₆₄ A ₁₇₆₈	N/A
	I-59	0.77 (N)	0.36	G ₁₈₉₆	N/A	C ₁₇₁₉	N/A
	I-69	0.55 (N)	0.29	G ₁₈₉₆	N/A	-	N/A
	I-89	0.78 (N)	0.30	A ₁₈₉₆ A ₁₈₉₉	N/A	T ₁₇₆₂ A ₁₇₆₄	N/A
	I-95	0.56 (N)	0.28	A ₁₈₉₆ A ₁₈₉₉	N/A	C ₁₇₁₉ T ₁₇₆₂ A ₁₇₆₄ T ₁₈₁₁	N/A
	I-105	1.25 (N)	0.79	A ₁₈₉₆ A ₁₈₉₉	N/A	C ₁₇₅₃ T ₁₇₆₂ A ₁₇₆₄	N/A
	I-177	0.62 (N)	0.39	A ₁₈₉₆	N/A	C ₁₇₂₁ A ₁₇₆₄	N/A
	adv	1.5 (N)	0.84	G ₁₈₉₆		C ₁₇₄₀	N/A
	ayw	4.21 (I)	1.74	G ₁₈₉₆		T ₁₆₇₈ C ₁₇₄₀	N/A

55

CLAIMS

1. Hepatitis B virus polynucleotide for use in evaluation of a hepatitis B disease state which comprises at least two of the following:

(i) a mutation in the Enhancer I region;

(ii) a mutation in the Negative Regulatory Element region;

(iii) a mutation in the Enhancer II/Core Upstream Regulatory Sequence/Basal Core Promoter region; and

(iv) a mutation which leads to an X peptide amino acid change to provide a cysteine or methionine residue;

the mutation being a variation from the normal nucleotide at that position in a respective one of HBV genotypes A to F.

2. Hepatitis B virus polynucleotide for use in detection of fulminant hepatitis B viral infection which comprises at least two of the following:

(i) a mutation at one or more of the following positions 1050, 1249 and 1250;

(ii) a mutation at one or more of the following positions 1633 and 1634;

(iii) a mutation at one or more of the following positions 1653, 1754, 1762, 1764, 1766, 1768, 1809, 1821, 1826 and 1838/9 insertion;

(iv) a mutation which leads to an X-peptide change to provide cysteine or methionine at one or more of amino acid positions 26, 72, 88, 127 and 130;

the mutations being variations from the normal nucleotide at that position in a respective one of HBV genotypes A to F.

3. Hepatitis B virus polynucleotide for use in detection of fulminant hepatitis B viral infection which comprises at least two of the following:

(i) a mutation which provides one or more of the following nucleotides at the following positions. G(1050), T(1249) and C(1250);

(ii) a mutation which provides one or more of the following nucleotides at the following positions.

A, T or C (1633) for genotype A or F

T, G or C (1633) for genotype B, C or D

A, T or C (1634) for genotype A, B, C or F

5 T, G or C (1634) for genotype D;

(iii) a mutation which provides one or more of the following nucleotides at the following positions.

A(1653), G(1754), T(1762), A(1764), T(1766), A(1768), T(1809), T(1821), A(1826), and AC (insertion between 1838 and 1839);

10 (iv) a mutation which leads to an X-peptide change to provide one of the following amino acids at the following amino acid positions.

C(26), M(88), M(130), M(127), M(141), M(5) and C(72).

4. Hepatitis B Virus polynucleotide for use in the detection of fulminant hepatitis B viral infection according to any of motifs 1 to 5 of Table 7 herein.

15 5. Hepatitis B virus polynucleotide for use in the detection of fulminant hepatitis B viral infection having at least two nucleotides in the positions according to any of numbers 1 to 31 in Table 8 herein.

6. Hepatitis B virus X-peptide having one of the following amino acids at the following amino acid positions:

C(26), M(88), M(130), M(127), M(141), M(5) and C(72).

20 7. Polynucleotide probe having a sequence complementary to that of any of claims 1 to 5.

8. Polynucleotide probe having a sequence complementary to a polynucleotide sequence coding for an HBV X-peptide of claim 6.

25 9. A test kit for detection of an HBV disease state which comprises polynucleotide fragment probes capable of hybridising under appropriate stringency conditions to any two of (i), (ii), (iii) and (iv) of claims 1, 2 or 3.

10. A test kit according to claim 7 comprising probes capable of hybridising to all four of (i) to (iv).

11. Antibody to any of the X peptides of claim 6.

5 12. An immunoassay for the detection of fulminant HBV which comprises an antibody to any of the X peptides of claim 6.

13. A test method for determining binding interactions between host or viral proteins and HBV polynucleotides which comprises;

- taking a polynucleotide fragment according to any of claims 1 to 6;
- 10 - applying said host or viral protein thereto; and
- determining the degree of binding between the protein and the polynucleotide fragment.

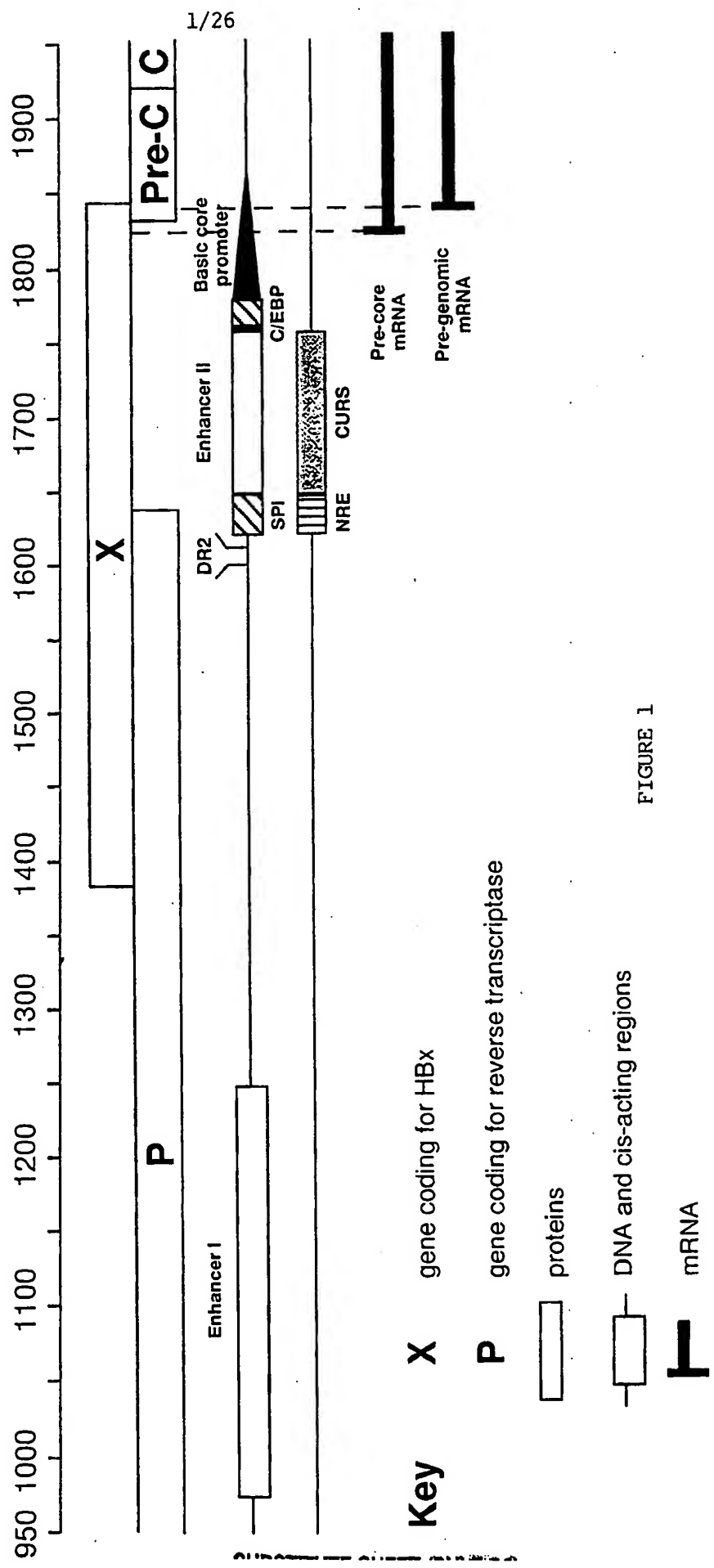


FIGURE 1

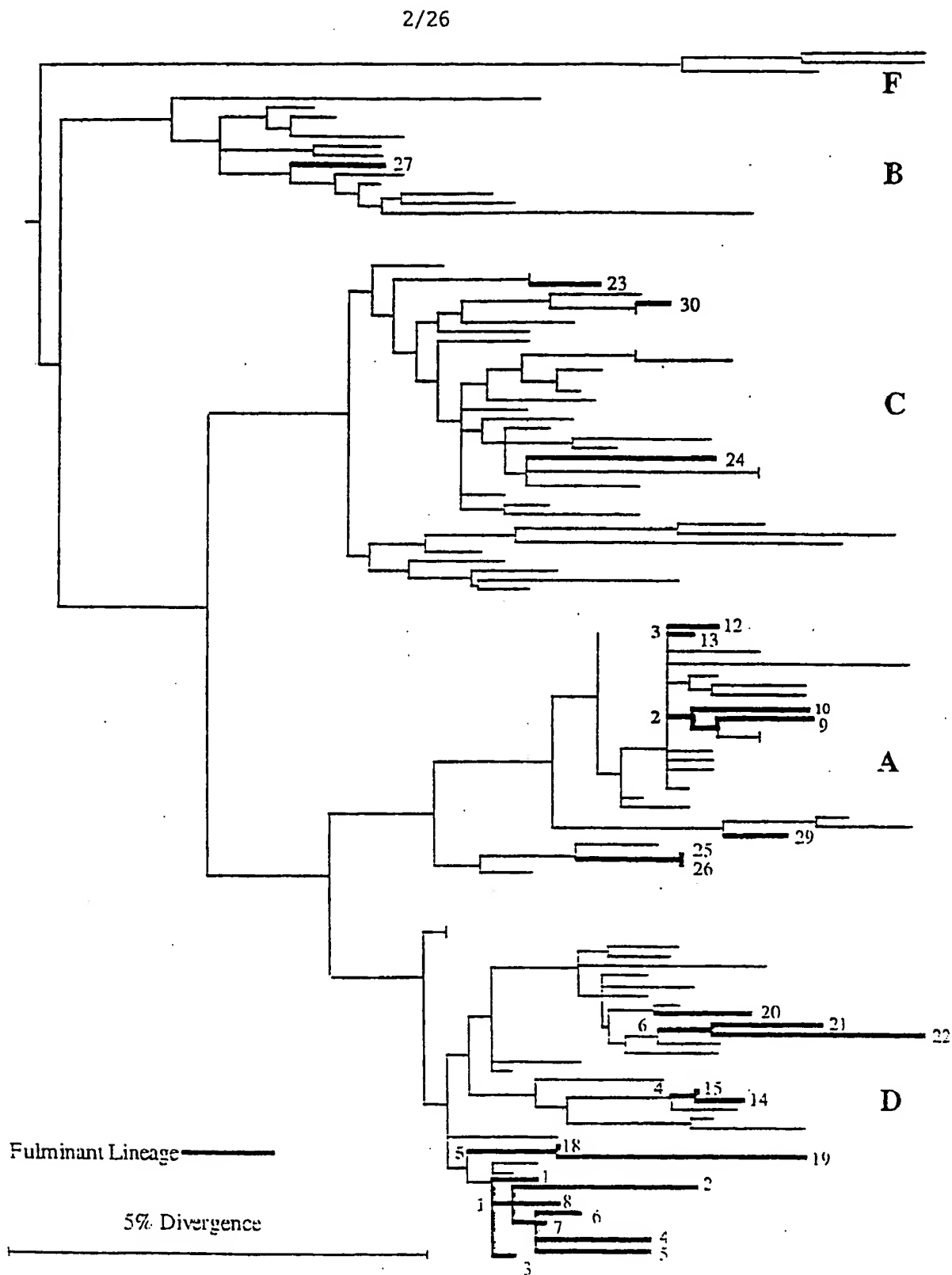
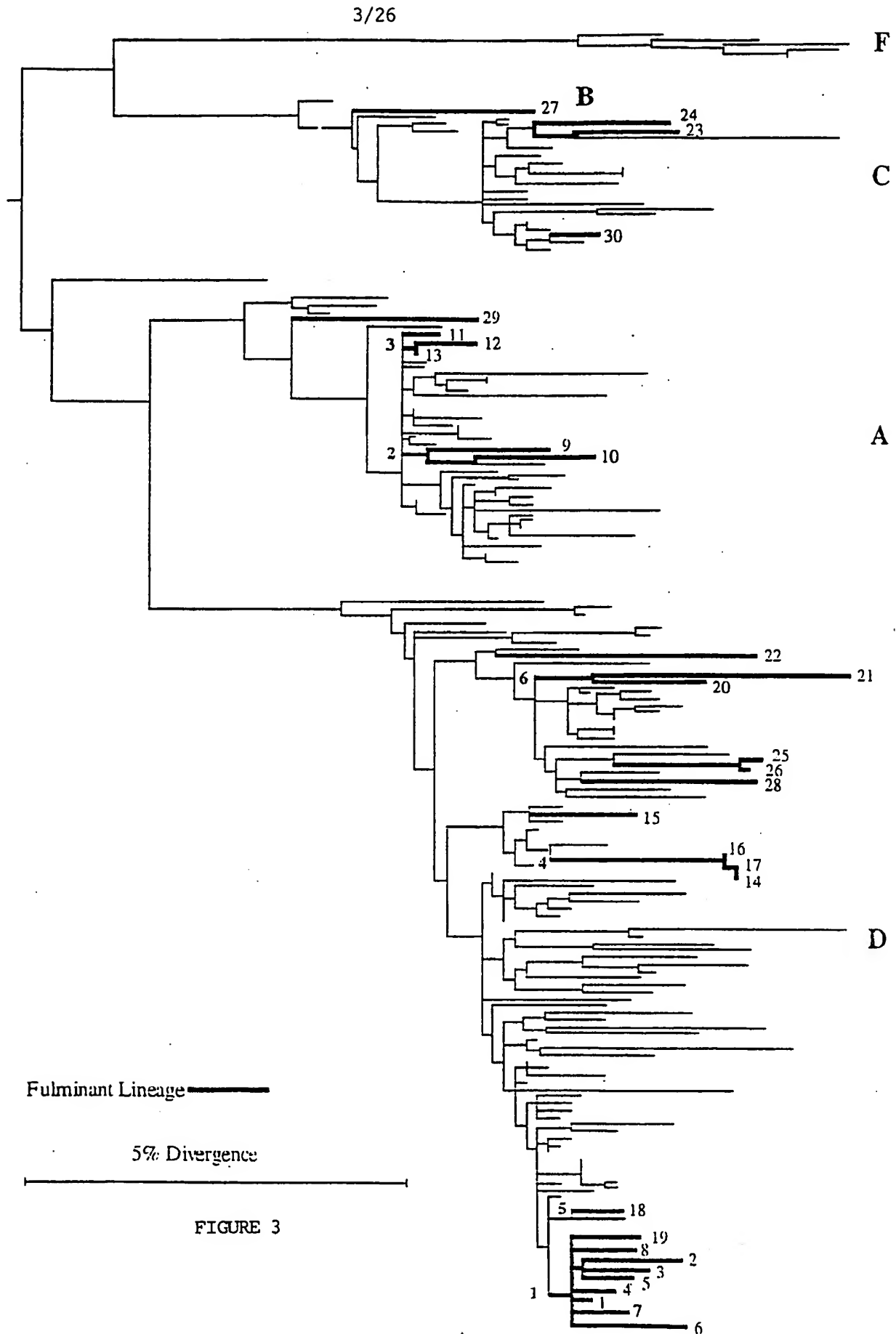


FIGURE 2



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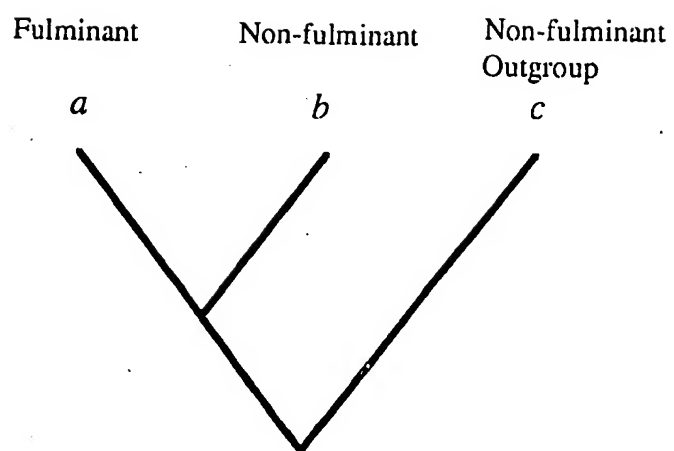


FIGURE 4

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Name	Sequences					

	1001					
HBVORFS	TGGGTCTTTT	GGGTTTTGCT	GCCCCTTTTA	CACAATGTGG	TTATCCTGCT	TTAATGCCCT
XXHEPAVT.
HBVGEN1A...T.
HBVAYWMCT.A..C..G....T.
HBVDNAT..A..GT.
XXHEPAVT.
HPBHBVAAA.....T.....
AHBV1C.....G
FHBV1C.....G
FHBV2	A.....GC
FHBV3A.....G
FHBV4G
FHBV5G.....C.....G
FHBV6C.....G
FHBV7G
FHBV12GC.....T.G..C..AGT.
FHBV13G
HBVP3CSX
HBVP2CSX
CHBV2G.....AAA...T.
HPBMUT	.A.....T.....A.....
FHBV14G.....
	1061					
HBVORFS	TGTATGCCTG	TATTCAATCT	AAACAGGCTT	TCACTTTCTC	GCCAACTTAC	AAGGCCTTTC
XXHEPAVA..G..GG.....	.T.....
HBVGEN1G..G.....
HBVAYWMCA..G.....
HBVDNAA..G..
XXHEPAVA..G..GG.....	.T.....
HPBHBVAAA..G.....
AHBV1G.....
FHBV1A..G.....
FHBV2A..G.....
FHBV3A..G.....	.T.....
FHBV4A..G.....
FHBV5A..G.....
FHBV6G.....
FHBV7	G.....A..G.....
FHBV12A..G.....
FHBV13A..G.....
HBVP3CSXG..A..T.....
HBVP2CSXG..A..T.....
CHBV2	C..C..AA.A.....
HPBMUTA..
FHBV14A..G.....
	1121					
HBVORFS	TGTGTAAACA	ATACCTGAAC	CTTTACCCCG	TTGCTAGGCA	ACGGCCAGGT	CTGTGCCAAG
XXHEPAVCC
HBVGEN1T.....CC
HBVAYWMCCC
HBVDNA	.AA.....	G..A.....C.....T...
XXHEPAVCC

FIGURE 5-1

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HPBHBVAA	CC.....C.....
AHBV1C.....	C.....CC.....
FHBV1CC.....
FHBV2CC.....
FHBV3CC.....
FHBV4G.....CCA.....
FHBV5CC.....
FHBV6C.....CC.....
FHBV7CC.....
FHBV12CC.....
FHBV13CC.....
HBVP3CSX
HBVP2CSX
CHBV2T.....CC.....
HPBMUTC.....CC.....
FHBV14CC.....W.....

1181

HBVORFS	TGTTTGCTGA	CGCAACCCCC	ACTGGCTGGG	GCTTGGTCAT	GGGCCATCAG	CGCATGCGTG
XXHEPAV
HBVGEN1
HBVAYWMC
HBVDNACATA
XXHEPAV
HPBHBVAA
AHBV1
FHBV1A.....A.....
FHBV2CG.....A.....
FHBV3
FHBV4
FHBV5
FHBV6
FHBV7T.....T.....
FHBV12C.TG.....
FHBV13C.....
HBVP3CSX
HBVP2CSX
CHBV2
HPBMUTG.....
FHBV14

1241

HBVORFS	GAACCTTTCT	GGCTCCTCTG	CCGATCCATA	CTGCGGAAC	CCTAGCCGCT	TGTTTGTCTC
XXHEPAVG.....
HBVGEN1C.....
HBVAYWMCG.....
HBVDNAGA.....
XXHEPAVG.....
HPBHBVAAA.....
AHBV1TC.....
FHBV1TC.....T.....T.....
FHBV2TC.....
FHBV3GC.....
FHBV4TC.....
FHBV5TC.....
FHBV6TC.....
FHBV7TC.....
FHBV12TC.....
FHBV13TC.....
HBVP3CSX

FIGURE 5-2

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HBVP2CSX
CHBV2C
HPBMUT
FHBV14TCC
1301						
HBVORFS	GCAGCAGGTC	TGGAGCAAAC	ATTCTCGGGA	CGGATAACTC	TGTTGTTCTC	TCCCGCAAAT
XXHEPAV
HBVGEN1G
HBVAYWMCTC..A
HBVDNAATC..AG..G
XXHEPAV
HPBHBVAAC
AHBV1ATC
FHBV1ATC
FHBV2ATC
FHBV3ATC
FHBV4ATC
FHBV5ATCN
FHBV6ATC
FHBV7ATC
FHBV12ATC
FHBV13ATC
HBVP3CSX
HBVP2CSXT
CHBV2CCC
HPBMUTA
FHBV14T
1361						
HBVORFS	ATACATCGTT	TCCATGGCTG	CTAGGCTGTG	CTGCCAACTG	GATCCTGCGC	GGGACGTCCT
XXHEPAVA
HBVGEN1
HBVAYWMCA
HBVDNAT..AT
XXHEPAVA
HPBHBVAAG
AHBV1A
FHBV1A.A
FHBV2T..AT.C
FHBV3A C
FHBV4A
FHBV5	N..TC...AT
FHBV6A ..G
FHBV7AT
FHBV12A
FHBV13A.A
HBVP3CSX
HBVP2CSX
CHBV2A
HPBMUTA
FHBV14
1421						
HBVORFS	TTGTTTACGT	CCCGTCGGCG	CTGAATCCCG	CGGACGACCC	TTCTCGGGGC	CGCTTGGGGA
XXHEPAVTAC
HBVGEN1AC
HBVAYWMC	.C.....AAC
HBVDNACAC
XXHEPAVTAC
HPBHBVAAAC

FIGURE 5-3.

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AHBV1	T	T	AC
FHBV1	T	T	AC
FHBV2	T	T	AC
FHBV3	T	T	AC
FHBV4	T	C	T	AC
FHBV5	T	T	T	AC
FHBV6	T	T	AC
FHBV7	T	T	AC
FHBV12	-----	-----	-----	-----	-----	-----	-----	-----
FHBV13	T	T	AC
HBVP3CSX
HBVP2CSX
CHBV2	AC
HPBMUTC	T	T	AC
FHBV14	AC

1481

HBVORFS	TCTTTCGTCC	CCTTCTCCGT	CTGCCGTTCC	GTCCGACCAC	GGGGCGCACC	TCTCTTTACG
XXHEPAV	...C	T	A
HBVGEN1	...C	T	A
HBVAYWMC	...C	T
HBVDNA	...C	AG
XXHEPAV	...C	T	A
HPBHBVAA	...C	T	A
AHBV1	...C	A
FHBV1	...C	A
FHBV2	...C	A
FHBV3	...C	A
FHBV4	...C	GT	A
FHBV5	...C	A
FHBV6	...C	A
FHBV7	...C	A
FHBV12	-----	-----	-----	A	C
FHBV13	...C	A
HBVP3CSX	T
HBVP2CSX	T
CHBV2	...C	T	A
HPBMUT	...C	T	A
FHBV14	...C	T	A

1541

HBVORFS	CGGACTCCCC	GTCTGTGCCT	TCTCATCTGC	CGGTCCGTGT	GCACTTCGCT	TCACCTCTGC
XXHEPAV	A
HBVGEN1	A
HBVAYWMC	A
HBVDNA	...T...A..
XXHEPAV	A
HPBHBVAA	A
AHBV1	A
FHBV1	A
FHBV2	A	T...T.T.T...
FHBV3	A	G
FHBV4	A
FHBV5	A	T
FHBV6	A
FHBV7	A
FHBV12	A
FHBV13	A
HBVP3CSX	A
HBVP2CSX	A

FIGURE 5-4

			9/26				
CHBV2	A.....
HPBMUT	A.....
FHBV14	A.....
	1601						
HBVORFS	ACGTCGCATG	GAGACCACCG	TGAACGCC--	-CACCAC TTC	TTGCCCAAGG	TCTTACATAA	
XXHEPAV	A.....	A.CA...
HBVGEN1	A.....
HBVAYWMC	A.....
HBVDNA	T..AAGT	C.....
XXHEPAV	A.....	A.CA...
HPBHBVAA	A.....
AHBV1	GGA.G
FHBV1	GAA.G
FHBV2	...T.....	GGA.G
FHBV3	GAA.G
FHBV4	GGA.G
FHBV5	GGA.G
FHBV6	GGA..
FHBV7	GGA.G
FHBV12	GGAA.	C.....	AC...
FHBV13	GGAA.	C.....
HBVP3CSX
HBVP2CSX
CHBV2	A.....
HPBMUT	GC	C..T.TA..	T....
FHBV14	A.....
	1658						
HBVORFS	GAGGACTCTT	GGACTCTCAG	CAATGTCAAC	GACCGACCTT	GAGGCATACT	TCAAAGACTG	
XXHEPAV	T.	T.....
HBVGEN1	T.	T.....
HBVAYWMC
HBVDNA	C..	C.....
XXHEPAV	T.	T.....
HPBHBVAA	T.	T.....
AHBV1	T.
FHBV1	T.
FHBV2	T.
FHBV3	T.
FHBV4	T.
FHBV5	T.
FHBV6	T.
FHBV7	T.	C.....
FHBV12	GT
FHBV13	TA
HBVP3CSX	T.	T.....
HBVP2CSX	T.
CHBV2	T.
HPBMUT	T.	T.....
FHBV14	T.	T.....
	1718						
HBVORFS	TTTGTTTAAG	GACTGGGAGG	AGTTGGGGGA	GGAGATTAGA	TTAAAGGTCT	TTGTACTAGG	
XXHEPAV	A	T....
HBVGEN1	A	G.....
HBVAYWMC	A	G.....	T.G.	T....
HBVDNA	G.....	C.....	G.....	T....
XXHEPAV	A	T....
HPBHBVAA	C.....	T.A...
AHBV1	A	G.G

FIGURE 5-5

			10/26				
FHBV1AGT
FHBV2A
FHBV3AG
FHBV4AG..G
FHBV5AG..G
FHBV6AG..G
FHBV7AG
FHBV12AG..G
FHBV13AG
HBVP3CSXAT.....
HBVP2CSXA
CHBV2C....T.A.T.	A....T....
HPBMUTAT.A....T.A.T.	A....T....
FHBV14AT.G.T....
	1778						
HBVORFS	AGGCTGTAGG	CATAAATTGG	TCTGCGCACC	AGCACCATGC	AACTTTTTC	A	CCTCTGCCTA
XXHEPAV
HBVGEN1
HBVAYWMC
HBVDNA
XXHEPAV
HPBHBVAA
AHBV1
FHBV1A
FHBV2
FHBV3
FHBV4
FHBV5
FHBV6
FHBV7
FHBV12
FHBV13
HBVP3CSX
HBVP2CSX
CHBV2
HPBMUT
FHBV14C.....
	1838						
HBVORFS	A-TCATCTCT	TGTTTCATGTC	CTACTGTTCA	AGCCTCCAAG	CTGTGCCTTG	GGTGGCTTTG	
XXHEPAV
HBVGEN1A
HBVAYWMC	A
HBVDNAC.....
XXHEPAV
HPBHBVAA	A
AHBV1
FHBV1	A
FHBV2	A
FHBV3
FHBV4	A
FHBV5
FHBV6
FHBV7
FHBV12A.....C.....
FHBV13
HBVP3CSX	A
HBVP2CSX
CHBV2	A

FIGURE 5-6

HPBMUTG	11/26A
FHBV14T.	A
1897						
HBVORFS	GGGCATGGAC	ATTGACCCTT	ATAAAGAATT	TGGAGCTACT	GTGGAGTTAC	TCTCATTTTT
XXHEPAVT....G....
HBVGEN1C....G....
HBVAYWMC	..A.....T....T..	A.....G..G....
HBVDNAG....
XXHEPAVT....G....
HPBHBVAA	..A.....T....G....C.
AHBV1C....G....A
FHBV1C....T....
FHBV2C....T..G....
FHBV3C....G....
FHBV4C....G....
FHBV5C....G....
FHBV6C....G....
FHBV7C....G....
FHBV12C....G....
FHBV13C....G....
HBVP3CSX	..A.....T....T....
HBVP2CSX
CHBV2	..A.....T....C....G....
HPBMUT	..A.....C....C....G....
FHBV14	..A.....T....G....
2017						
HBVORFS	GCCTTCTGAC	TTTTTTCCTT	CGGTACGAGA	TCTTCTAGAT	ACCGCCTCAG	CTCTGTATCG
XXHEPAVC....A....A....
HBVGEN1C....A....C....
HBVAYWMCC.A....A....A....
HBVDNAC....C...A....C....C
XXHEPAVC....A....A....
HPBHBVAAC....A....A....
AHBV1C....A.G....G....
FHBV1CC....A....
FHBV2C....A....G....
FHBV3C.C....A....
FHBV4C....A....
FHBV5C....A....
FHBV6C....A....
FHBV7C....A....
FHBV12C....A....
FHBV13C....A....GA....C
HBVP3CSXCA....C....C....
HBVP2CSXC....
CHBV2C....G	TCA....
HPBMUTC....C.C....T....
FHBV14A....C....C....A....
2017						
HBVORFS	GGATGCCTIA	GAGTCTCCTG	AGCATTGTTC	ACCTCACCAT	ACTGCACTCA	GGCAAGCAAT
XXHEPAV	..A.....
HBVGEN1	..A.....
HBVAYWMC	..A.....A..T..
HBVDNA	A..A.....C..C..
XXHEPAV	..A.....
HPBHBVAA	..A.....	..A.....
AHBV1	..A.....
FHBV1	..A.....

FIGURE 5-7

			12/26			
FHBV2	...A.....					
FHBV3	...A.....		...G.....			
FHBV4	A..A.....					
FHBV5	...A.....					...C..
FHBV6	...A.....					
FHBV7	...A.....					
FHBV12	...A.....			...CA..		
FHBV13	...A.....		...AC.....			
HBVP3CSX						
HBVP2CSX			...A.....			
CHBV2	...A.....	...A.....				
HPBMUT	...A.....					
FHBV14	...A.....					
2077						
HBVORFS	TCTTTGCTGG	GGGGAATAA	TGACTCTAGC	TACCTGGGTG	GGTGTTAATT	TGGAAGATCC
XXHEPAV	A..G.....				...G.....	
HBVGEN1						
HBVAYWMC		...T..		C.....	...G.....	...C.....
HBVDNA	...C.....	...G.....		AT.....	...A.....	
XXHEPAV	A..G.....				...G.....	
HPBHBVAA	...C.....	...T..	A.....T	C.....	...G.....	
AHBV1						
FHBV1						...A.....
FHBV2						
FHBV3						
FHBV4	...A.....				...A.....	
FHBV5		...C.....			...C.....	
FHBV6						
FHBV7						
FHBV12						
FHBV13						
HBVP3CSX	A.....	...CT..	...A.....	...A.....		...C.....
HBVP2CSX	C.....					...C.....
CHBV2			...G.....	C.....	...G.....	
HPBMUT	...G.....	...TG..	...A.....		...GC.....	
FHBV14	...A.....	...T..		C.....	...G.....	
2137						
HBVORFS	AGCATCTAGG	GACCTAGTAG	TCAGTTATGT	CAACACTAAT	ATGGGCCTAA	AGTTCAGGCA
XXHEPAV	.AT...C...					...A.....
HBVGEN1		...T..	.T.....		...T..	
HBVAYWMC	.A...C...			T.....		
HBVDNA	...G.....			T.....		...A.....
XXHEPAV	.AT...C...					...A.....
HPBHBVAA	.A.....			T.....		
AHBV1	...A.....					
FHBV1	...A.....					
FHBV2	...A.....	...A.AT..		...AT..		
FHBV3	...A.....					
FHBV4	...A.....					
FHBV5	...A.....					
FHBV6	...A.....					
FHBV7	...A.....					
FHBV12	...A.....					
FHBV13	...A.....					
HBVP3CSX		...A.....	...A.....	T.....		
HBVP2CSX				T.....		
CHBV2	...T...C...			T.....C...	...C.....	...T.....
HPBMUT	.A...C...	...A.T..	...A.....		...T..G.	

FIGURE 5-8

			13/26			
FHBV14	.A....C...T.....C.	T.....T.....		
	2197					
HBVORFS	ACTATTGTGG	TTTCACATT	CTTGTCTCAC	TTTTGGAAGA	GAAACAGTCA	TAGAGTATTT
XXHEPAVT.
HBVGEN1	...T.....G....
HBVAYWMCG....G.....
HBVDNAT..A.C..T..G..T..AC	.T..A.....
XXHEPAVT.
HPBHBVAAG....
AHBV1	...C.....C..T.
FHBV1	...C.....C..T.
FHBV2	...T.....C..T.
FHBV3	...T.....C..T.
FHBV4	...C.....C..TC
FHBV5	...C.....C..T.
FHBV6	...M.....T..A.C..Y..G..T..AC	.C..A.....
FHBV7	...C.....C..T.
FHBV12	...C.....T.
FHBV13	...C.....C..T.
HBVP3CSXG....
HBVP2CSX	...C.....G..T.
CHBV2T..T..AC	AT..T....
HPBMUTTC..
FHBV14Y..T.T	G.....G..C	.T.....
	2257					
HBVORFS	GGTGTCTTTC	GGACTGTGGA	TTCGCACTCC	TCCAGCTTAT	AGACCACCAA	ATGCCCTAT
XXHEPAVT
HBVGEN1
HBVAYWMCTA.....
HBVDNA	...C.....C..
XXHEPAVT
HPBHBVAAT
AHBV1
FHBV1
FHBV2
FHBV3
FHBV4A.....
FHBV5
FHBV6	...C.....
FHBV7
FHBV12	A.....
FHBV13
HBVP3CSXA.....
HBVP2CSX
CHBV2TT....
HPBMUTA.....
FHBV14
	2317					
HBVORFS	CTTATCAACA	CTTCCGGA	CTACTGTTGT	TAGACGA---	---CGAGGCA	GGTCCCCTAG
XXHEPAVG.
HBVGEN1G.
HBVAYWMCG.	..TG.....
HBVDNAG..A..
XXHEPAVG.
HPBHBVAAG.
AHBV1	.C.....
FHBV1	.C.....
FHBV2	.C.....

FIGURE 5-9

			14/26			
FHBV3	.C.					
FHBV4	.C.					
FHBV5	.C.					
FHBV6	.C.					
FHBV7	.C.					
FHBV12	.C.					
FHBV13	.C.			G.		
HBVP3CSX			A.			A.
HBVP2CSX		G.		A.		
CHBV2		G.	GG.A.A.	A.CGG	GAC.	
HPBMUT		G.				
FHBV14		G.	A.			
2371						
HBVORFS	AAGAAGAACT	CCCTCGCCTC	GCAGACGAAG	ATCTCAATCG	CCGCGTCGCA	GAAGATCTCA
XXHEPAV				G.		
HBVGEN1						
HBVAYWMCG				G.		
HBVDNA				G.		
XXHEPAV				G.		
HPBHBVAA				G.	A.	A.
AHBV1				G.		
FHBV1				G.		
FHBV2				G.		A.
FHBV3		A.		G.	A.	GAG.
FHBV4			G.			
FHBV5		G--				
FHBV6				G.		
FHBV7				G.		
FHBV12				G.		
FHBV13				G.		
HBVP3CSX						
HBVP2CSX						
CHBV2			C.			
HPBMUT			C.	GC.		
FHBV14				G.		
2431						
HBVORFS	ATCTCGGGAA	TCTCAATGTT	AGTATTCCTT	GGACTCATAA	AGTGGGTAAC	TTTACGGGGC
XXHEPAV					G...A...	
HBVGEN1					G...A...	A...
HBVAYWMCG					G...A...	
HBVDNA					G...G...	T...
XXHEPAV					G...A...	
HPBHBVAA			C.		G...A...	
AHBV1					G...G...	T..T.
FHBV1						
FHBV2						
FHBV3		C.			G...G...	T..T.
FHBV4						
FHBV5						
FHBV6					G...G...	T..T.
FHBV7						
FHBV12						
FHBV13						
HBVP3CSX						
HBVP2CSX		T.			G...A...	
CHBV2		C.			G...A...	
HPBMUT		G.			G...A...	
FHBV14		C.			G...A...	

FIGURE 5-10

2491

HBVORFS	TTTATTCCTC
XXHEPAVT..
HBVGEN1T..
HBVAYWMCT..
HBVDNAT..
XXHEPAVT..
HPBHBVAAT..
AHBV1T..
FHBV1	-----
FHBV2	-----
FHBV3T..
FHBV4	-----
FHBV5	-----
FHBV6T..
FHBV7	-----
FHBV12	-----
FHBV13	-----
HBVP3CSXT..
HBVP2CSXT..
CHBV2T..
HPBMUTT..
FHBV14T..

FIGURE 5-11

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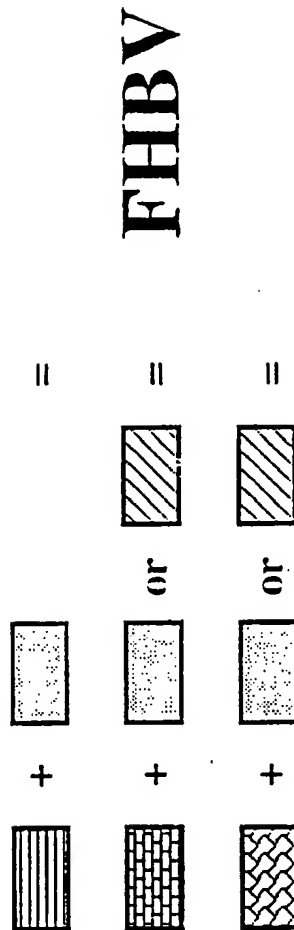
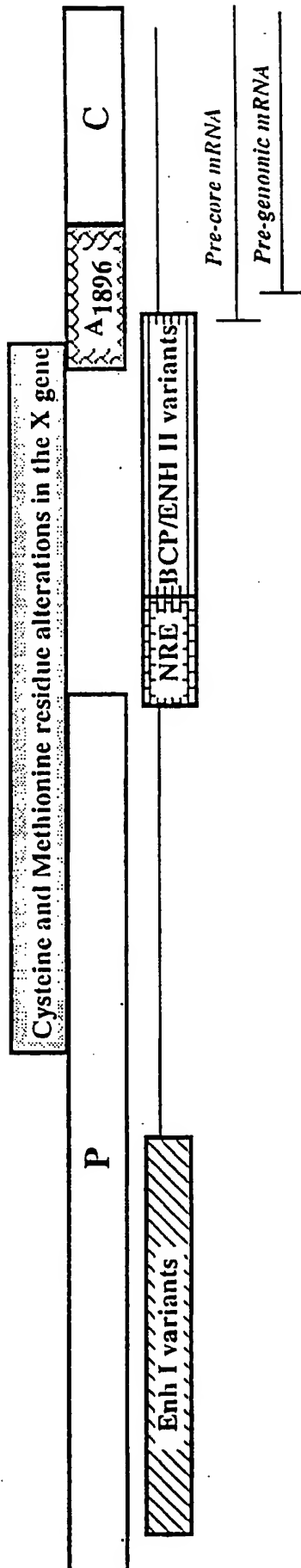


FIGURE 6

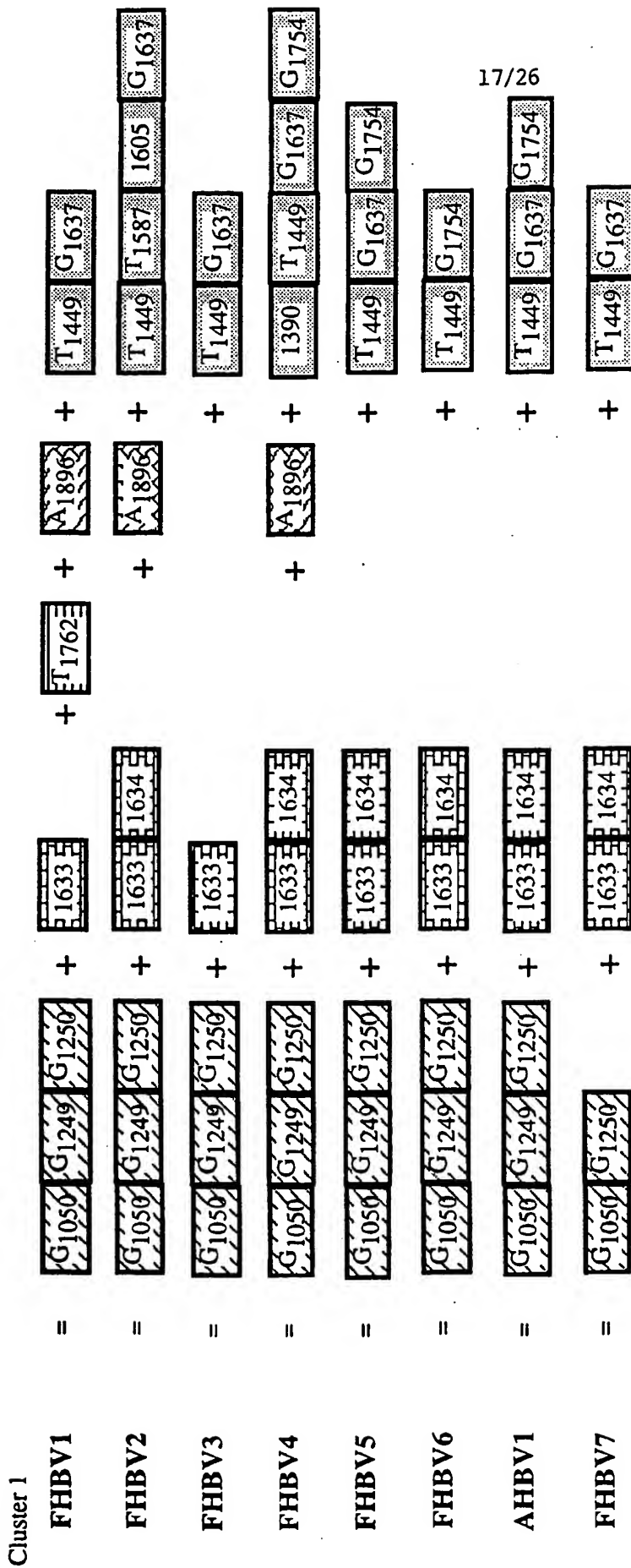
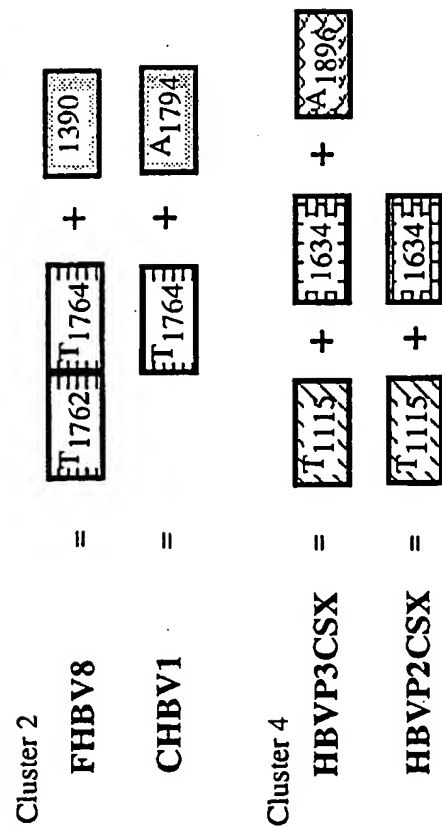


FIGURE 6-2



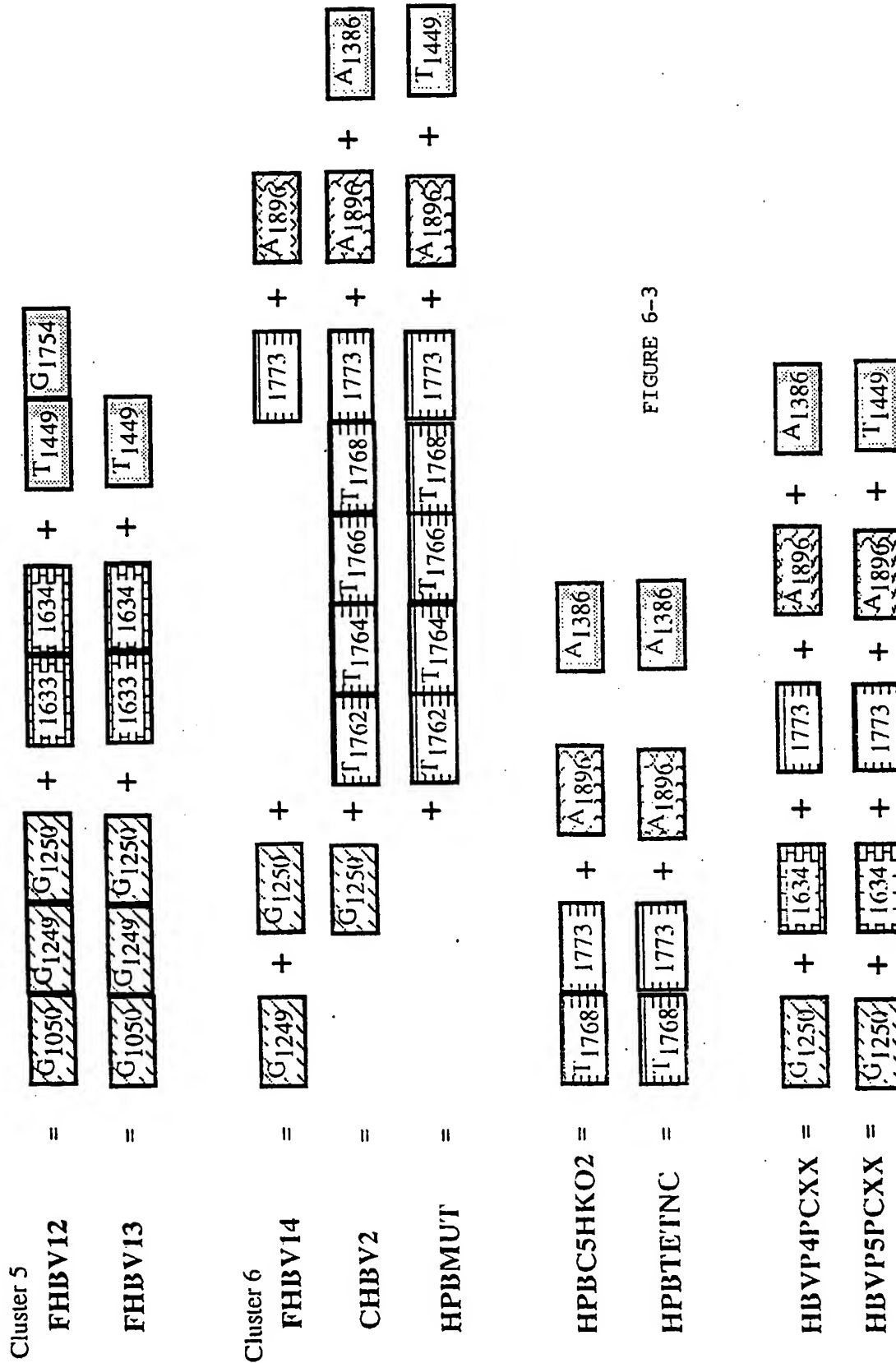


FIGURE 6-3

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	1643		1686
	↓ (CURS Starts)		↓ (Enh-II starts)
Con-ayw	CAAGGTCTTACATAAGAGGACTCTTGGACTCTCTGTAATGTCAACGACCGACCTTGAGGCATACTT		
Con-adw	-----C-A-C-----C-----		
FHBV-1	-----C-----		
FHBV-4	-----AC-----		
FHBV-5	-----AC-----		
FHBV-8.1	-----		
FHBV-8.5	-----T-----C-A-C-----T-----		
CHBV-1	-----C-A-C-----C-----		
FHBV-9	-----C-A-C-----C-----		
FHBV-12	-----A-----C-A-C-----T-----		
FHBV-14	-----		
CHBV-2	-----C-----		
FHBV-15	-----		
FHBV-16	-----C-A-C-----T-----		
I-40	-----C-----		
I-59	-----G-----C-----		
I-69	-----CG-----C-----		
I-89	-----T-----C-----		
I-95	-----G-----C-----		
I-105	-----G-----C-----		
I-177	-----G-----C-----		

	1743
	↓ (CURS ends, BCP starts)
Con-ayw	CAAAGACTGTTTGTTTAAAGACTGGGAGGAGCTGGGGGAGGAGATTAGATTAAAGGTCTTTGTATT
Con-adw	-----G-----G-----G-----
FHBV-1	-----T-----G-----T-----C-----
FHBV-4	-----T-----C-----T-A-T-A-----
FHBV-5	-----G-----T-----C-----T-A-T-A-----
FHBV-8.1	-----T-----T-----G-----
FHBV-8.5	-----G-----G-----A-----C-----G-----T-----T-----
CHBV-1	-----G-----G-----G-----
FHBV-9	-----G-----G-----G-----
FHBV-12	-----G-----T-----G-----
FHBV-14	-----G-----T-----C-----T-G-----
CHBV-2	-----G-----T-----C-----T-A-T-A-----C-----
FHBV-15	-----G-----T-----C-----C-----
FHBV-16	-----G-----T-----
I-40	-----AT-----T-----XXXXXXXXXX-A--A-----
I-59	-----C-----T-----C-----
I-69	-----T-----C-----
I-89	-----T-----T-A-----C-----
I-95	-----T-----T-A-----C-----
I-105	-----T-----C-----T-A-----C-----
I-177	-----C-----T-----A-----C-----

FIGURE 7-1

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	1776
	↓ (Enh-II ends)
Con-ayw	AGGAGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATGCAACTTTTTTCACCTCTGCCTAA--TC
Con-adw	-----C-----
FHBV-1	-----A-----
FHBV-4	-----T-----
FHBV-5	-----T-----
FHBV8.1	-----
FHBV-8.5	-----
CHBV-1	-----A-----
FHBV-9	-----
FHBV-12	-----C-----T-----C-----
FHBV-14	-----C-----A-----
CHBV-2	-----T-----
FHBV-15	-----AC-----
FHBV-16	-----A-----T--T-----C-----
I-40	-----
I-59	-----
I-69	-----
I-89	-----
I-95	-----T-----
I-105	-----
I-177	-----

	1849	Genotype	1896	1899
	↓ (End of BCP)			
Con-ayw	ATCTCTTGT	D	G	G
Con-adw	-----	A	G	G
FHBV-1	-----	D	A	G
FHBV-4	-----	D	A	A
FHBV-5	-----	D	A	A
FHBV-8.1	----T----	D	A	A
FHBV-8.5	-----	A	G	G
CHBV-1	-----	A	G	G
FHBV-9	-----	A	G	G
FHBV-12	-----	D	G	G
FHBV-14	----T----	D	A	A
CHBV-2	-----	D	A	A
FHBV-15	-----	D	A	G
FHBV-16	-----	A	G	G
I-40	-----	D	G	G
I-59	-----	D	G	G
I-69	-----	D	G	G
I-89	-----	D	A	A
I-95	-----	D	A	A
I-105	-----	D	A	A
I-177	-----	D	A	G

FIGURE 7-2

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FIG. 8.A

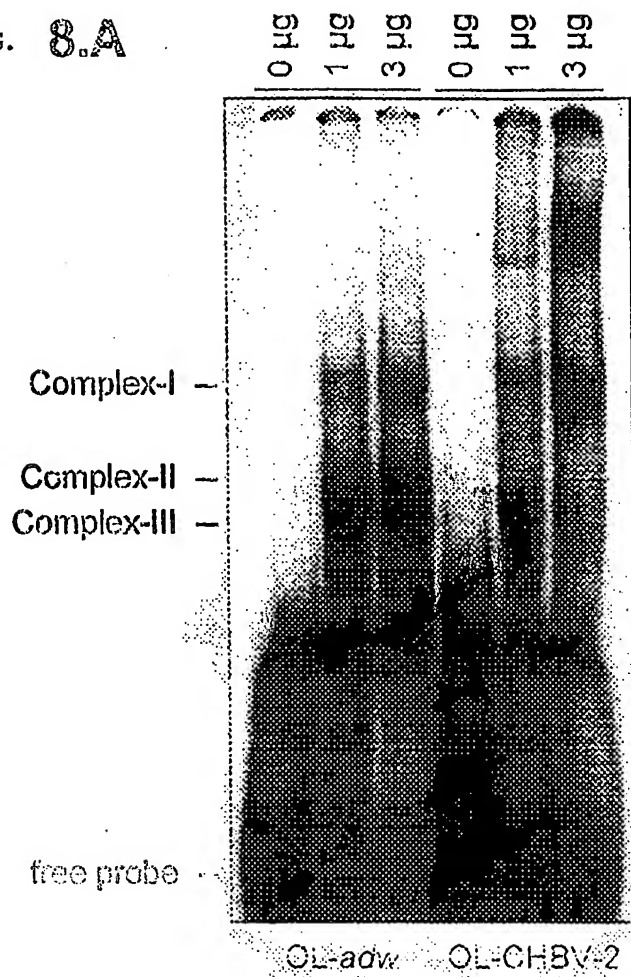
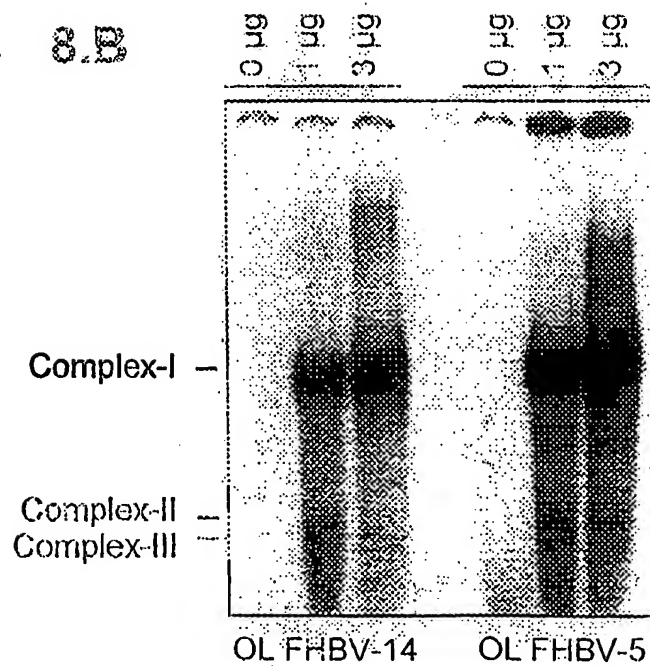


FIG. 8.B



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FIG. 8.C

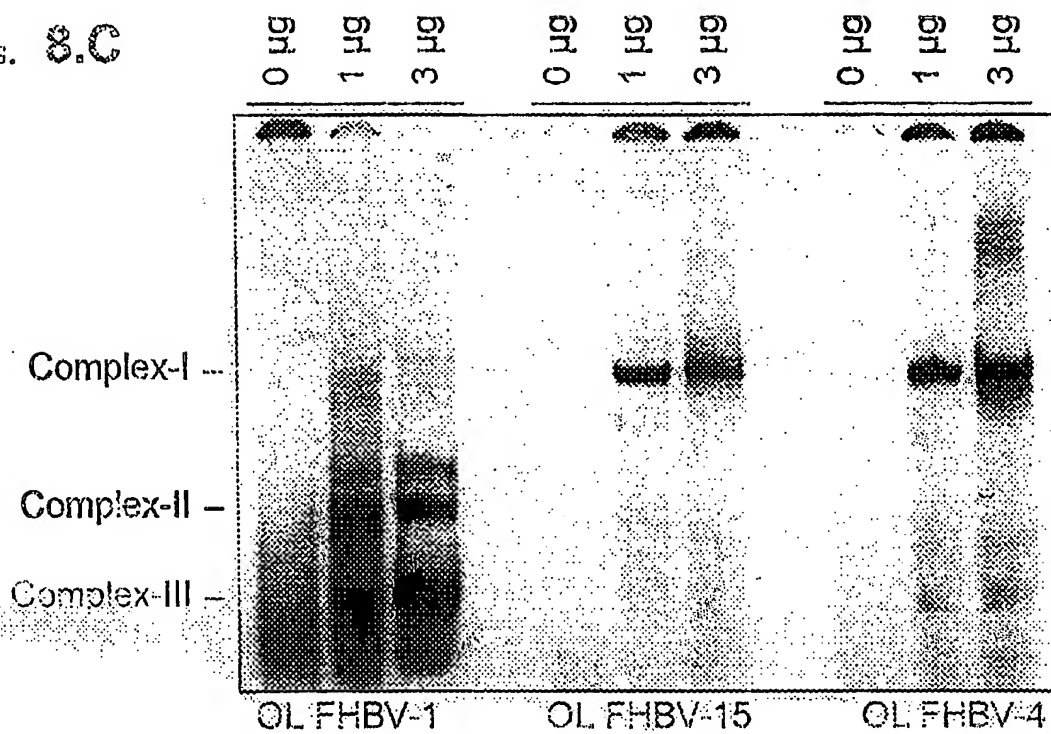
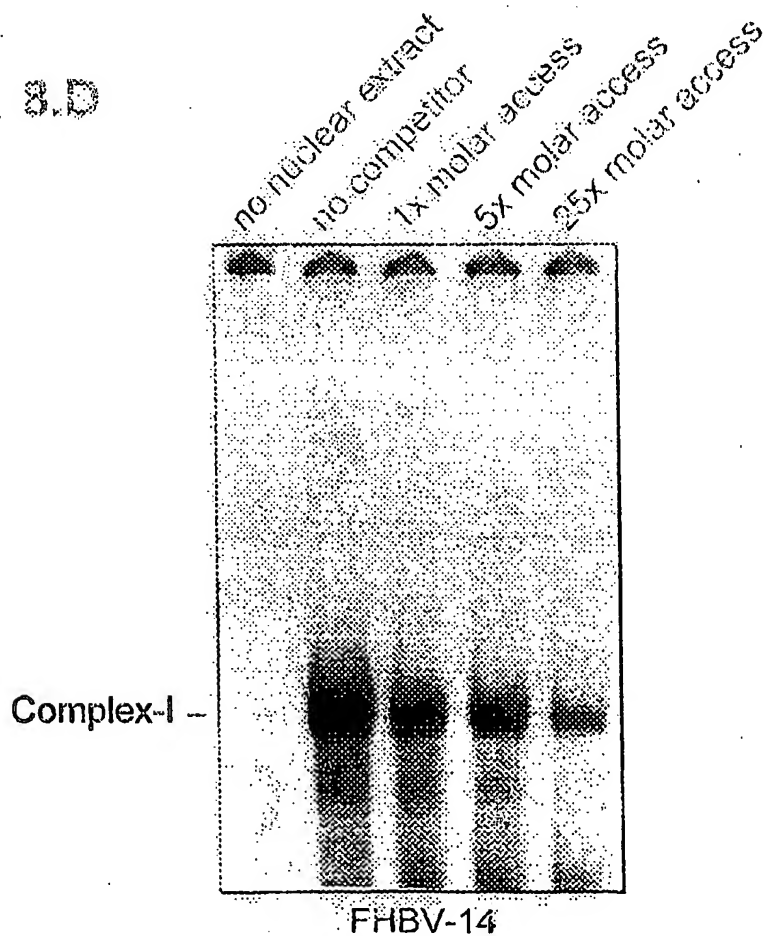


FIG. 8.D



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FIG. 9.A

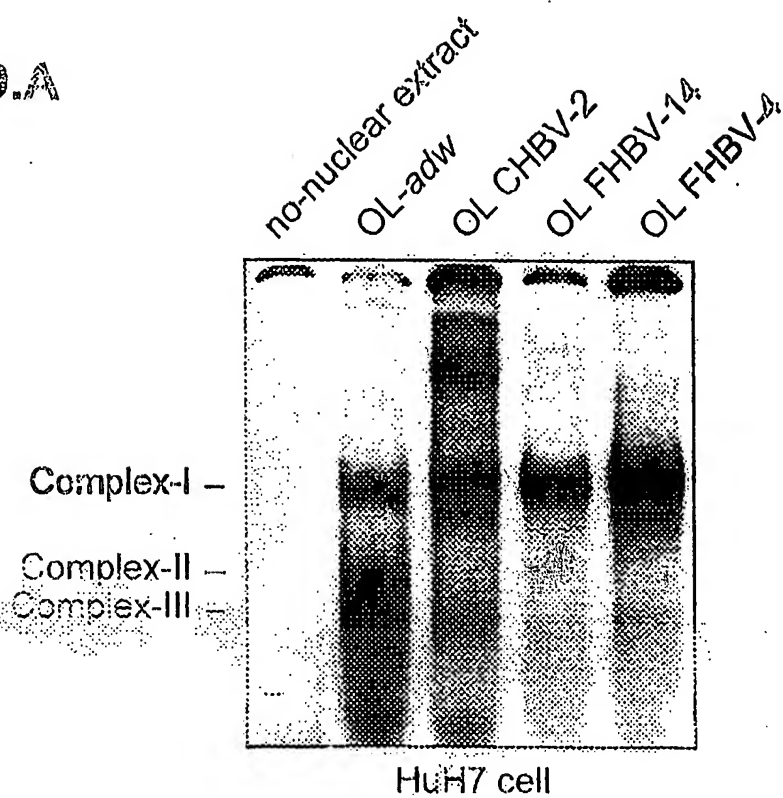
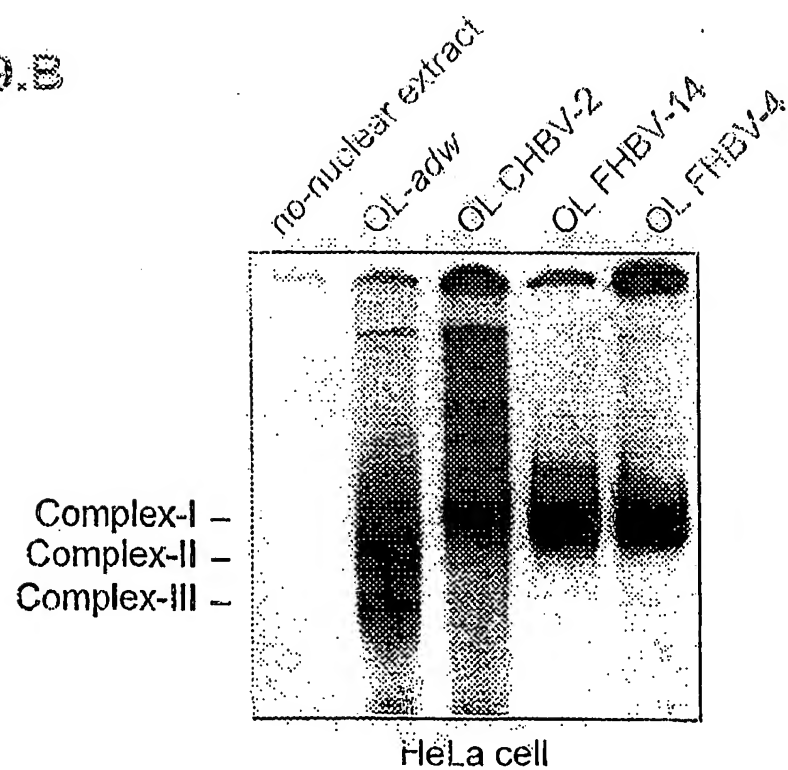


FIG. 9.B



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Cases	Luciferase value $\times 10^6$	Variations in the BCP
Con- <i>adw</i>	1.50	
FHBV-1	11.51	T ₁₇₆₂ A ₁₈₂₆
FHBV-4	8.81	C ₁₇₅₃ A ₁₇₅₇ T ₁₇₆₂ A ₁₇₆₄ , T ₁₇₆₆ A ₁₇₆₈ T ₁₈₁₀
FHBV-5	10.25	C ₁₇₅₃ A ₁₇₅₇ T ₁₇₆₂ A ₁₇₆₄ , T ₁₇₆₆ A ₁₇₆₈ T ₁₈₁₀
FHBV-14	11.39	A ₁₇₅₇ T ₁₇₆₄ G ₁₇₆₆ A ₁₈₃₄ T ₁₈₄₅
CHBV-2	9.68	C ₁₇₅₃ A ₁₇₅₇ T ₁₇₆₂ A ₁₇₆₄ A ₁₇₆₈ C ₁₇₇₁ T ₁₈₁₀
FHBV-15	7.98	T ₁₆₇₈ C ₁₇₅₂ A ₁₇₅₇

FIGURE 10-1

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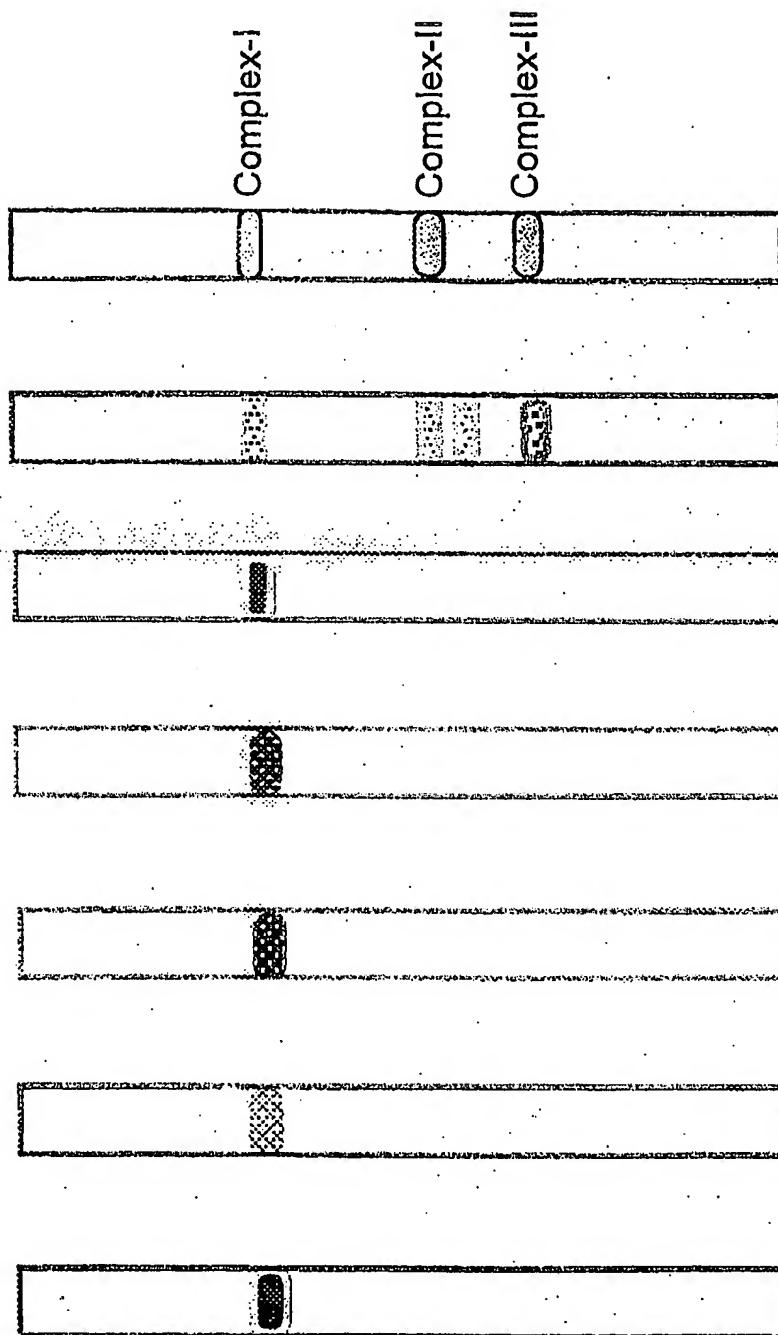
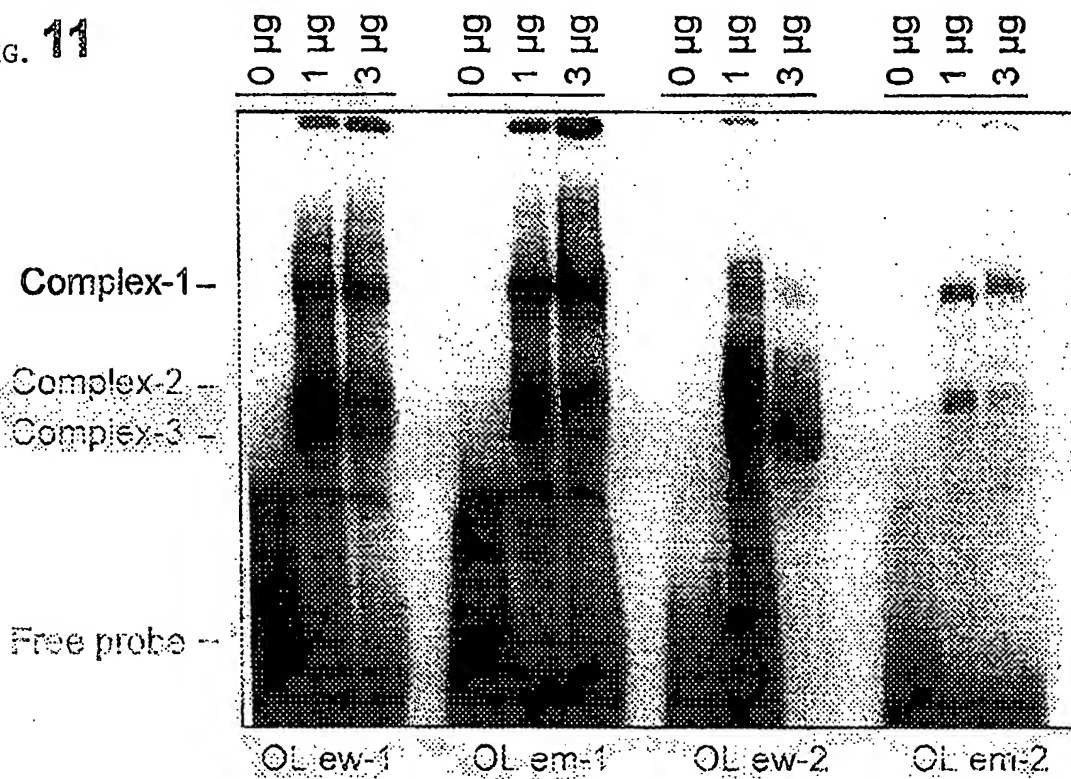


FIG 10 -2

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FIG. 11



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